

AN ABSTRACT OF THE THESIS OF

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Both trout and mammals are susceptible to nitrosamine-induced carcinogenesis. In mammals, cytochrome P450 2E1 was responsible for the N,N'-dimethylnitrosamine (NDMA) activation which then lead to initiation of carcinogenesis. The first aim of our study was to determine if an ortholog of cytochrome P450 2E1 was present in rainbow trout liver. The p-nitrophenol (PNP) hydroxylase activity which is one of the enzyme activities associated with P450 2E1 was not detectable when 50 mg/kg imidazole was used as an inducer. Both 100 mg/kg and 200 mg/kg imidazole killed the fish. When fish were exposed in 0.25 % ethanol there was no induction of PNP hydroxylase activity. Fish then were exposed to 0.5 % ethanol for 8 hr, 16 hr, and 24 hr. PNP hydroxylase activity was slightly elevated with exposure time in female trout but not in males. The average PNP hydroxylase activity was only about 10 pmole/min/mg microsomal protein, a value only one-eighth of that seen in control rat liver microsomes. Inbred trout liver had much higher PNP hydroxylase activity than that of

albino or outbred trout liver. In the Western blot analysis, all of the microsomes isolated from trout livers, had two or three protein bands which migrated in the cytochrome P450 region when rabbit anti-rat P450 2E1 antibodies was used as a probe. However, no correlation was observed between the PNP hydroxylase activity and the protein concentrations as determined by immunoassay. Evidence was obtained that these liver proteins were mainly P450 LMC1 and LMC2 together with a smaller quantity of a new P450 isozyme designated X1, with an approximated M_r of 52,000. Previous studies have shown that sexually mature male trout liver had higher level of P450 LMC2 than mature female trout liver. In our studies, there was no significant difference in PNP hydroxylase activity between male and female trout livers. These data imply that NDMA activation probably does not occur via P450 LMC2. Whether other P450 isozymes might be responsible for the enzyme activity in the rainbow trout liver remains to be uncovered. There was inconsistency of Western blot analysis data between kidney and liver microsomes when anti-trout P450 LMC2 and anti-rat P450 2E1 antibodies were used as probes. The results implied the presence of a P450 isozyme, designated X2, which comigrated with P450 LMC2 ($M_r = 54,000$). This isozyme might be in the liver but absent in the kidney and cross-reacted with anti-rat P450 2E1 antibody. The other possibility was, in the sexually mature trout kidney, the isozyme highly cross-reacted with anti-trout P450 LMC2 antibody but poorly recognized by anti-rat P450 2E1 antibody.

Several researchers have shown that in mammalian systems, glutathione S-transferase π (GST- π) was a useful biomarker for preneoplastic lesions in chemical carcinogenesis. Recently, GST from trout liver was isolated in our laboratory and was classified as π family according to the N-terminal amino acid sequences and its dimeric composition. We, then, wanted to investigate if this GST isozyme was a biomarker for preneoplastic foci in chemical induced carcinogenesis in rainbow trout. Rainbow trout fry were exposed to 0.5 ppm

aflatoxin B₁ (AFB₁) for 30 minutes and then transferred to clean water. Every two weeks after exposure, livers were removed from the fish and either pooled and homogenized for biochemical studies or fixed in formalin for immunohistochemical studies. Small increases of cytosolic and microsomal GST activity occurred with age. However, no difference of total GSTs activity was found in the livers between control and AFB₁-treated fish. Immunohistochemical studies using the avidin-biotin-complex (ABC) method showed that the polyclonal antibodies against trout GST- π detected the preneoplastic lesions and neoplasms in the livers of AFB₁-treated rats. The cytoplasm and some nuclei in untreated trout liver parenchymal cells were stained positively by these same antibodies. Proximal tubules in both untreated and treated trout kidney reacted strongly with the GST- π antibodies. However, no GST- π was expressed in regenerating cells in treated trout liver. Unlike mammals, therefore, GST- π was not expressed in preneoplastic nodules and neoplasms in AFB₁-treated trout liver.

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Studies on Carcinogen Metabolizing Enzymes in the Rainbow Trout

INTRODUCTION

Chemical carcinogenesis comprises at least two or three stages: (1) initiation; an irreversible process, during which specific genes or protooncogenes are altered. (2) promotion; a reversible step in which the initiated cells proliferate and might become the precursors of malignant cells. (3) progression; an irreversible stage which refers to malignant conversion of benign lesions (Pitot and Sirica, 1980).

Rainbow trout (Oncorhynchus mykiss) have been used extensively in chemical carcinogenesis studies. Several carcinogens effective in mammalian systems, such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), benzo[a]pyrene (BP), aflatoxin B₁ (AFB₁) were able to elicit neoplasms in rainbow trout. Among these chemicals, MNNG is a direct-acting carcinogen which induces neoplasms without metabolic activation (Hendricks et al., 1980), while BP and AFB₁ are procarcinogens which require metabolic activation to produce tumors (Hendricks et al., 1985; Wales et al., 1978). Xenobiotic biotransformation processes involved in the metabolic activation of carcinogens are generally catalyzed by phase I enzymes such as cytochrome P450 which inserts one atom of atmospheric oxygen into their substrates. Electrophilic phase I products can either attack cellular macromolecules (DNA, RNA, protein) to initiate mutagenicity and carcinogenicity or become the substrates of phase II enzymes such as glutathione S-transferases (GSTs) which conjugate the substrates with thio groups and make the compounds more water-soluble for excretion.

Nitrosamines, a group of widely occurring carcinogens, are known to require metabolic activation for conversion to their carcinogenic and cytotoxic forms. The activation

process, generally involving the oxygenation of the α -carbon, has been shown to be a cytochrome P450-dependent reaction (Lai and Acros, 1980; Yang et al., 1985b). In mammals cytochrome P450 2E1 is the major enzyme responsible for the dimethylnitrosamine (NDMA) demethylase activity at very low NDMA concentration (Yang et al., 1985a). There is much evidence showing that in mammals P450 2E1 is induced by pretreatment with a variety of compounds such as ethanol, acetone, imidazole, pyridine, isopropanol, etc. (Tu et al., 1983; Koop and Coon, 1984; Koop et al., 1985; Yang et al., 1985b; Patten et al., 1986; Kim et al., 1988; Ueng et al., 1991).

Teleosts are susceptible to toxicity and carcinogenicity induced by nitrosamines (Ashley and Halver, 1968; Grieco et al., 1978; Nakazawa et al., 1985; Ding and Lam, 1989; Marty et al., 1990; Kaplan et al., 1991). However, none of the P450 isozymes purified from fish have been reported to be involved in nitrosamine activation, although several monooxygenase catalytic activities have been identified and characterized (Gooch and Matsumura, 1983; Funari et al., 1987; Schell et al., 1987; Stegeman and Kloepper-Sams, 1987; Buhler and Williams, 1989; Stegeman, 1989; Miranda et al., 1989; Miranda et al., 1990 a and b). Recently, Kaplan et al. (1990) suggested that the immunoreactive microsomal protein designated P450 pj in the viviparous fish, Poeciliopsis, from desert streams of northwestern Mexico, recognized by polyclonal antibodies against rat P450 2E1, might be equivalent to rat cytochrome P450 2E1. These researchers synthesized a 49 base-oligomer, complementary to bases of the rat P450 2E1 sequence and used it as a probe for Northern blots. They detected a 3.3 kb mRNA and found that ethanol treatment of the fish both induced and suppressed this mRNA, depending on concentration and exposure time. However, they did not report any direct evidence for induction of the P450 2E1 ortholog, either by measuring enzyme activity or by direct

measurement of enzyme levels. No other studies have appeared indicating that an ortholog of cytochrome P450 2E1 is present in other teleost species or that this isozyme is inducible in fish. Since rainbow trout are relatively sensitive to nitrosamine-induced carcinogenicity (Ashley and Halver, 1968; Grieco et al., 1978), the first aim of our study was to examine the possibility of inducing cytochrome P450 2E1 in trout.

Chemical carcinogenesis in fish is, like that in other vertebrates, a multistep process (Hendricks et al., 1984). In general, treatment with different hepatocarcinogens produces a common phenotypic alteration in the biochemistry of enzymes such as glucose-6-phosphate dehydrogenase, glucose-6-phosphatase, adenosine triphosphatase and -glutamyl-transpeptidase, in isolated foci of initiated cells (enzyme-altered foci) and in hepatic nodules (Hinton et al., 1988). Several investigators have shown that in mammals these foci typically exhibit decreased levels of cytochrome P450 and increased levels of phase II xenobiotic metabolizing enzyme activities (Roomi et al., 1985; Kunz et al., 1987; Tsuda et al., 1988). Among these latter enzymes, the most consistent marker of preneoplasia in chemical-induced liver cancer is the $\text{Pi}(\pi)$ -class of glutathione S-transferase (GST- π) which is a cytosolic homodimeric (YfYf) enzyme (Muramatsu et al., 1987; Power et al., 1987; Eimoto et al., 1988; Ito et al., 1988). Loranžana et al. (1987) demonstrated that the immunohistochemical staining for cytochrome P450 LMC2 (a constitutive P450 isozyme in trout, now designated P450 2K1) was reduced in AFB₁-treated trout hepatoma nodules and that of cytochrome P450 1A1 (a polycyclic aromatic hydrocarbon inducible P450) in trout, was absent both in normal and hepatoma nodules. Therefore, we were interested in determining whether the possible expression of GST- π in normal trout liver and in AFB₁-induced preneoplastic foci is similar to that seen in mammalian systems.

Chapter 1.

Evidence for an Ortholog of Rat Cytochrome P450 2E1 in Livers of Untreated and Chemically Exposed Rainbow Trout

ABSTRACT

The purpose of this study was to determine if an ortholog of cytochrome P450 2E1, an isozyme which is responsible for the metabolic activation of nitrosamines and resulting carcinogenesis in mammals, is present in rainbow trout liver. Two chemicals, imidazole and ethanol, have been used in this study. Rainbow trout were treated with three doses of imidazole, 50 mg/kg, 100 mg/kg and 200 mg/kg. The two higher doses killed the fish. No detectable p-nitrophenol (PNP) hydroxylase activity, which is a measure of mammalian P450 2E1 activity, was observed in the 50 mg/kg-treated group. Rabbit anti-rat P450 2E1 IgG was used as a probe of the trout liver microsomes in Western blots. The intensity of the cross-reaction bands recognized by these P450 2E1 antibodies was higher in 0.5 % ethanol-treated fish than 0.25 % ethanol-treated group. Therefore, 0.5 % ethanol was chosen for a time course study. Both male and female rainbow trout were exposed by immersion in a solution of 0.5 % ethanol for 8 hours, 16 hours, and 24 hours, respectively. The PNP hydroxylase activity in the trout liver microsomes was about 10 pmole/min/mg protein, only one-eighth of that found in untreated rats. No significant difference in PNP hydroxylase or in total cytochrome P450 content was observed in microsomes from ethanol-treated male trout liver. The hepatic microsomes of female trout, however, showed a small continuous increase in PNP hydroxylase activity with exposure time. Significant induction of total P450 content in females also was seen after 16 hours treatment with ethanol. Western blot analysis with rabbit anti-rat P450 2E1 antibodies detected two and

occasionally three protein bands in microsomes isolated from rainbow trout. The two major bands comigrated with the trout cytochrome P450 constitutive forms LMC1 and LMC2 while the third weaker band, designated P450 X1 (M_r of about 52,000), migrated between these two P450s. No induction of these immunoreactive proteins, and no correlation between the PNP hydroxylase activity and immunoreactive protein quantitation was observed. Thus no direct evidence was shown in the Western blots that the equivalent of mammalian P450 2E1 was present in rainbow trout. PNP hydroxylase activities of three strains (albino, inbred, outbred) of rainbow trout liver microsomes were also measured. The enzyme activity from inbred strain was two-fold and three-fold higher than that from albino and outbred strains, respectively. Western blot analysis detected one major band (P450 X1) which comigrated with rat P450 2E1 in microsomes isolated from albino, and inbred trout strains, but not from the outbred trout strain. P450 LMC1 and LMC2 bands also were detected in microsomes from inbred and outbred trout. However, there was no correlation between PNP hydroxylase activity and immunoreactive protein quantitation in liver microsomes from these three strains.

Expression of trout liver P450 LMC2 is known to be much higher in sexually mature male trout than in females. Although PNP hydroxylase activities were slightly higher in sexually mature trout than in two-year old fish, no significant difference in activity was observed between sexually mature males and females. The low PNP hydroxylase activity of sexually mature male trout known to contain high concentrations of P450 LMC2 suggests that this isozyme was not responsible for the nitrosamine activation seen in the rainbow trout. Western blot analysis of kidney microsomes from sexually mature trout showed only a light band compared to that seen in liver microsomes from the same animals when probed with rabbit anti-rat P450 2E1 antibodies. Since kidney microsomes of sexually mature male trout are known to contain

a much higher P450 LMC2 concentrations than that of liver microsomes from the same animals, these findings suggest the presence of a fourth P450 isozyme, designated X2 ($M_r = 54,000$), that is recognized by the anti-rat P450 2E1 antibodies.

INTRODUCTION

A multitude of structurally diverse physiological substrates, for example, steroids, fatty acids as well as xenobiotic compounds (drugs, haloalkanes, carcinogens, and pesticides) are metabolized in various animal tissues by cytochrome P450 mixed-function oxidase systems (Guengerich, 1987). Rabbit cytochrome P450 3a and rat P450j can be induced by a variety of dissimilar chemicals such as ethanol, acetone, pyridine, isoniazid, imidazole, etc. (Koop and Coon, 1984; Porter et al., 1989; Ryan et al., 1986). In the standardized nomenclature (Nebert, 1991), this isozyme has been designated as cytochrome P450 2E1. This P450 isozyme has an important role in human health as a result of being readily induced by acute and chronic alcohol ingestion (Coon et al., 1984). Increased hepatotoxicity of acetaminophen (Tylenol) has been reported in human alcoholics and ethanol-treated animals (Goldfinger et al., 1978; Peterson et al., 1980; Sato et al., 1981). Morgan et al. (1983) showed the P450 2E1 induced by ethanol highly contributes to the toxicity of acetaminophen in rabbit. Cytochrome P450 2E1 and 1A2 catalyzed nearly all of the acetaminophen activation in human liver microsomes (Raucy et al., 1989). In addition, some chemicals used in industry such as carbon tetrachloride and various carcinogenic nitrosamines such as N,N-dimethylnitrosamine (NDMA), N,N-diethylnitrosamine (NDEA), and N-nitroso-2,6-dimethylmorphine (NNDM) are metabolized by P450 2E1 to elicit toxicity (English and Anders, 1985; Kokkinakis et al., 1985). Rabbit P450 2E1 efficiently catalyzed the demethylation of NDMA at very low

substrate concentration (Yang et al., 1985a and b). Rat P450 2E1 was also the primary microsomal catalyst of NDMA activation at substrate concentration relevant to hepatocarcinogenesis induced by NDMA (Thomas et al., 1987). Koop et al. (1989) reported that P450 2E1 acts as a p-nitrophenol hydroxylase which converts p-nitrophenol (PNP) to 4-nitrocatechol. Aniline is also a substrate of P450 2E1 (Patten et al., 1986; Song et al., 1986). Therefore, NDMA demethylase, PNP hydroxylase and aniline hydroxylase all have been used in the routine assay for P450 2E1.

Cytochrome P450 2E1 is expressed not only in liver but also in extrahepatic tissues. Hepatic parenchymal cells contain the highest concentration of P450 2E1 which is distributed mainly in the centralobular region (Forkert et al., 1991; Böhler et al., 1992). Recently, Koop et al. (1991) demonstrated that P450 2E1 was immunochemically detectable at low levels in Kupffer cells from untreated rats and was induced greater than 10-fold by acetone-treatment of the animals. Cytochrome P450 2E1 was also immunochemical detectable in nasal and kidney microsomes but not in brain, lung, adrenal, heart, intestine, ovary, spleen, testis, and uterus from untreated or ethanol-treated rabbits (Ding et al., 1986; Ueng et al., 1987). However, Waziers et al. (1989) found P450 2E1 was expressed in human and rat intestine, although the concentration is much lower than that in liver. Hansson et al. (1990) showed hepatic P450 2E1 is constitutively expressed in rat brain.

Although P450 induction commonly is thought of as being a response to exogenous chemicals, P450 2E1 induction constitutes a good example of a system that also can respond to altered endogenous physiologic states. Imaoka et al. (1990) demonstrated starvation can alter the amount of P450s in rat hepatic microsome. P450 2E1 in the rat was induced 2.5-fold by a 48-hr starvation and its increase reflected the increase of metabolic activity of hepatic microsomes toward aniline,

7-ethoxycoumarin, and NDMA. Tu and Yang (1983) also reported that fasting in the rat induces nitrosamine dealkylase, a catalytic activity now associated with P450 2E1. Fasting had a pronounced synergistic effect on acetone-dependent induction of P450 2E1 mRNA and apo-P450 2E1 by two-fold (Johansson et al., 1988). Bellward et al. (1988) reported that the diabetic state leads to an increase in hepatic aniline hydroxylation and an induction of hepatic cytochrome P450 2E1 in the rat.

Multiple mechanisms appear to operate in P450 2E1 induction and these do not primarily involve increased transcription. In rats treated with a single intragastric dose of acetone, there were rapid rises in the microsomal levels of P450 2E1 and in PNP hydroxylation, but no changes were observed in mRNA levels of P450 2E1 (Ronis et al., 1991). Song et al. (1987) found that stabilization of P450 2E1 mRNA appears to be the major mechanism of enzyme induction during streptozotocin-induced diabetes in rat. However, Johansson et al. (1990) demonstrated that transcription of the P450 2E1 gene is activated by starvation, indicating that this P450 gene is under transcriptional control for certain physiological conditions.

As seen in mammals, nitrosoamines have been found to induce hepatic carcinomas in teleosts, such as rainbow trout (*O. mykiss*), medaka (*Oryziops latipes*) and *Poecillio* (Ashley and Halver, 1968; Grieco et al., 1978; Schultz and Schultz, 1984). Cytochrome P450s are the primary catalysts for xenobiotic monooxygenase activity in fish. Distinct forms of P450 have been identified in marine teleosts, such as scup (*Stenotomus chrysops*), cod (*Gadus morhua*) and in the freshwater teleost, rainbow trout. Fish P450s are induced by certain foreign compounds especially polyaromatic hydrocarbons (PAHs) (Buhler and Williams, 1989; Stegeman, 1989). Five constitutive cytochrome P450s have been purified from rainbow trout designated LMC1 to LMC5 (Miranda et al., 1989) and a rainbow trout P450 1A1 induced by PAHs also has been isolated

(Williams and Buhler, 1982). Kaplan et al. (1991) suggested the differential induction of tumors among genotypes of Poecilliopsis exposed to N-nitroso-diethylamine (NDEA) may, in part, be due to differences in liver P450 pj activity (the piscine equivalent of mammalian P450 2E1). These researchers also demonstrated that P450 pj mRNA was induced by 0.3 % ethanol when fish were exposed for 24 hours but was suppressed by treatment with 0.5 % ethanol for 48 hours. This suggests that regulation of P450 pj expression in Poeciliopsis is different from that of rat P450 2E1. In rats, ethanol treatment increases P450 2E1 activity, but not the concentrations of mRNA (Thomas et al., 1987). Since trout is very susceptible to NDMA carcinogenicity, there might be similar metabolism of NDMA in trout as in mammals. The aim of this study is to determine if cytochrome P450 2E1 equivalent is present in rainbow trout and if it is inducible by the inducers of P450 2E1.

MATERIALS AND METHODS

A. Chemicals

Imidazole, p-nitrophenol, 4-nitrocatechol, NADPH, and ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO); BCA protein assay reagents and rabbit anti-sheep IgG polyclonal antibodies were from Pierce Chemicals Co. (Rockford, IL); and 95 % ethanol was purchased from Department of Chemistry, OSU. [¹²⁵I]protein A was obtained from ICN Biomedicals, Inc. (Irvine, CA). Other chemicals were all reagent grade and commercially available.

B. Treatment of Animals

1. **Imidazole Treatment-** Mt. Shasta strain rainbow trout (Oncorhynchus mykiss) (200-700 g) were anesthetized with MS

222 then dosed with 50 mg/kg, 100 mg/kg, and 200 mg/kg imidazole through i.p. injection. Imidazole was dissolved in saline (0.9 % NaCl) and pH was adjusted to 7.5. All fish were sacrificed 16 hours after treatment.

2. Ethanol Treatment- Rainbow trout were starved overnight then immersed in 0.25 % and 0.5 % ethanol prepared from 95 % ethanol. There were three fish in each tank containing 15 gallons ethanol solution. Air stones were put in the tank and bubbled continuously. Fish were sacrificed 17 hours later. However, only the liver microsomes from fish exposed in 0.5 % ethanol showed a treatment effect. We, therefore, decided to study the time course response at the 0.5 % ethanol concentration. In order to get maximum induction of P450 2E1, rainbow trout (200-450 g) were starved for three days and then exposed in 30 gallons of 0.5 % (v/v) ethanol prepared from 95 % ethanol (six fish per treatment) with continuous air bubbling. The temperature of the ethanol solution was 15.5°C. Eight, sixteen and twenty four hours later, fish were sacrificed.

3. Untreated Animals- In order to study the strain and sex difference in PNP hydroxylase activity and in protein detected by immunoblotting, two year-old albino trout (a genetically recessive trait in a strain of trout lacking pigmentation), inbred trout (the crossing of closely related trout) and outbred trout (the crossing of genetically unrelated trout) weighing between 200 to 400 g, and three years-old Mt. Shasta sexually mature male and female trout (1000-2000 g) were sacrificed without treatment.

4. Pyridine Treatment on the Rat- 200-250 g male Sprague-Dawley rats were i.p. with 100 mg/kg pyridine daily for four days. The animals were fasted for 24 hours before being sacrificed.

C. Preparation of Hepatic Microsome and Cytosol

Livers were finely minced and homogenized in 3 volume of Tris-acetate buffer [0.1 M Tris-acetate, pH 7.5, 0.1 mM EDTA, 0.1 mM phenylmethanesulphonylfluoride(PMSF)]. Microsomes were obtained by centrifugation at 10,000 g for 30 min followed by 100,000 g for 90 min. The final supernatant was cytosol. The microsome pellets were resuspended in 0.1 M potassium phosphate, pH 7.25, 20 % glycerol, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 0.1 mM PMSF and frozen in aliquots at -80°C.

D. Enzyme Assays

The catalytic activity of microsomal P450 2E1 was measured by p-nitrophenol (PNP) hydroxylation (Koop et al., 1989). Reaction mixtures (final volume of 1 ml) contained 0.1 M potassium phosphate buffer, pH 6.8, 0.1 mM PNP, 1 mM ascorbic acid, 1 mM NADPH and 2 mg microsomal protein. The PNP mixtures were incubated at 27°C for one hour. The reactions were quenched with 0.2 ml of 1.5 N perchloric acid. The resulting 4-nitrocatechol, stabilized by ascorbate, was determined spectrally at 510 nm in 1.0 ml of the supernatant after the addition of 0.1 ml of 10 N NaOH. An extinction coefficient of $14.5 \text{ mM}^{-1}\text{cm}^{-1}$ was determined for 4-nitrocatechol for the conditions used.

E. Other Assays

Protein concentration was determined by using BCA assay (Smith et al., 1985). Bovine serum albumin was diluted in the same buffer as the sample when constructing a standard curve.

Ethanol sample solutions were collected before and after exposing fish and then analyzed enzymatically. The concentration of ethanol was measured by the absorbance at 340

nm resulting from the appearance of NADH after incubation with alcohol dehydrogenase and aldehyde dehydrogenase in potassium diphosphate buffer, pH 9.0 and NAD (Boehringer Mannheim standard kit).

Total microsomal cytochrome P450 was determined from the CO-reduced difference spectrum as described (Omura and Sato, 1964).

F. Immunoblot

Microsomal proteins were separated on a 8 % SDS-polyacrylamide gel and electroblotted to nitrocellulose membrane. The blots were incubated in 5 % solution of powdered milk in Tris buffer saline with Tween 20 (TBS-Tween) followed with 50 μ l/ml rabbit anti-rat cytochrome P450 2E1 polyclonal antibody (OXYgene Dallas) in 25 ml TBS-Tween buffer or 10 μ g/ml rabbit anti-trout cytochrome P450 LMC1 and LMC2 at 4°C overnight. After washing in TBS-Tween buffer for four times (5 min/each), the blots were incubated in [125 I]protein A at room temperature for 1 hour. Visualization was performed by autoradiography. The immunoconjugates were quantified by scanning the autoradiography on a laser densitometer. Sheep anti-rabbit P450 2E1 antibody (a gift from Dr. Koop) diluted to 1:5000 also were tested on the blots. After washing in TBS-Tween, the blots were incubated with rabbit anti-sheep IgG (1:500) antibody at room temperature for 1 hour followed with [125 I]protein A. Because the latter antibody gave a high background on the film, we decide to choose rabbit anti-rat P450 2E1 antibody as a probe in the following Western blot analysis.

G. Statistical Analysis

The statistical significance of differences was evaluated by the Student's-t-test. A p value of 0.05 was considered as

statistically significant.

RESULTS

A. Imidazole Treatment

The fish dosed with 100 mg/kg and 200 mg/kg imidazole were dead when examined 16 hours after treatment. Thus we were only able to isolate the hepatic microsomes from fish injected with 50 mg/kg and the saline control. Microsomal PNP hydroxylase activity was determined for these fish, but when only 0.5 mg protein was incubated with the substrate at 25°C for 1 hour, enzyme activity was not detectable. Western blot analysis showed two bands cross-reacted with rabbit anti-rat P450 2E1, but no evidence for induction was observed.

B. Ethanol Treatment

The most common inducer used for P450 2E1 in mammals is ethanol. When PNP hydroxylase activity of liver microsomes from trout exposed to 0.25 % and 0.5 % ethanol were examined no evidence for induction of enzyme activity was seen. However, in the Western blots, one of the two bands which yielded a cross-reaction with the antibodies was more intense in the 0.5 % ethanol group than in the 0.25 % ethanol group. According to these preliminary tests, we, therefore, chose the 0.5 % ethanol concentration to dose the trout in the time course response study.

The actual concentration of ethanol before and after exposing fish was measured (see Materials and Methods) and the data are shown in Table 1-1. Since it was not possible to continuously monitor the true concentration of ethanol in the approximately 30 gallon tanks, we determined the ethanol concentration at the start and at the end of the exposure period using a kit from Boehringer Mannheim Co. No ethanol was

detectable in the water from the untreated group. Although the manner of preparation of the ethanol solution for each group was the same, there was apparent deviation from the nominal 0.5 % ethanol concentration and the 16 hr-treatment fish were exposed to a higher concentration of 0.86 % (6.88 g/l) compared to other groups. The ethanol concentration decreased somewhat during exposure except for the 8 hr-treatment group. All of the fish exposed to 0.5 % ethanol survived except for one that died in the 24 hours treatment group.

The total P450 content of male and female trout microsomes are reported in Fig. 1-1 and Fig. 1-2, respectively. There were no significant differences in P450 content in relation to exposure time in male trout. However, in the female group, the 16 hour-treatment fish demonstrated the highest P450 content and there was a small increase in total cytochrome P450 with increasing exposure time. Trout hepatic PNP hydroxylase activity (10 pmole/min/mg) was much lower than that of rat liver microsomes from untreated (0.86 nmole/min/mg) and pyridine-exposed (9.28 nmole/min/mg) animals. No significant induction of the PNP hydroxylase activity was observed in the treated male trout liver microsomes (Fig. 1-3). However, in females, enzyme activity increased slightly with exposure time and the fish immersed in ethanol for 24 hours had the highest enzyme activity (Fig. 1-4).

Microsomes are routinely suspended and stored in 20 % glycerol at -80°C . Several pieces of evidence have shown that glycerol is oxidized to formaldehyde in the rat primarily by P450 2E1 (Winters and Cederbaum, 1990). Glycerol can, therefore, serve as a competing substrate for P450 2E1. Although no reports about the effect of glycerol on the PNP hydroxylase activity were found, we hypothesized that the activity could be inhibited by glycerol. When we used freshly prepared, glycerol-free microsomes from untreated fish, however, we did not see any increase in PNP hydroxylase

activity.

Upon use of sheep anti-rabbit P450 2E1 antibody in Western blots, only one protein was detected, which comigrated with trout P450 LMC1 (Fig. 1-5). However, there were two major protein bands recognized by rabbit anti-rat P450 2E1 antibodies in every fish hepatic microsome preparation examined (Fig. 1-6 and 1-7). There was an additional protein in a few microsome samples that comigrated with rat P450 2E1. The two major proteins comigrated with the trout hepatic constitutive cytochrome P450 LMC1 ($M_r=50,000$) and LMC2 ($M_r=54,000$). No induction of these two bands in the 0.5 % ethanol treatment group was found using immunoquantitation and no correlation was found between PNP hydroxylase activity and enzyme concentration as determined by immunoquantitation. A third faint band appeared in some samples, comigrating with rat P450 2E1 between the trout P450 LMC1 and LMC2 bands. This new band, presumably a previously unknown P450 with an approximately $M_r=52,000$, has been designated P450 X1.

The relative intensity between the two major bands was altered when repeat Western blot experiments were run with microsomes that had been stored frozen (Fig. 1-6). Since these two protein bands comigrated with trout P450 LMC2 and LMC1, we had hypothesized that these proteins were these P450 isozymes which were recognized by rabbit anti-rat P450 2E1. Figure 1-8 showed that microsomes from pyridine-treated rats had highly induced level of P450 2E1 upon probing with antibodies against this isozyme. The rabbit anti-rat P450 2E1 also immunoreacted with purified trout P450 LMC1 and LMC2. Therefore, every trout liver microsome sample was also probed with rabbit anti-trout P450 LMC1 and LMC2. As evidence that there is a significant homology between LMC1 and LMC2 (Miranda et al., 1990b), every sample showed at least two bands in Western blots when probed with anti-LMC1 IgG and anti-LMC2 IgG. However, no significant induction of these P450s was noted in the ethanol-treated fish (Fig. 1-9 and Fig. 1-10).

The hepatic PNP hydroxylase of inbred trout showed the highest activity and was significantly different from that of albino and outbred trout (Fig. 1-11). No difference was found in this enzyme activity between albino and outbred trout. When Western blots were probed with anti-rat P450 2E1 antibody, only one major protein band was detected which comigrated with rat P450 2E1 in the albino and inbred trout liver microsomes (Fig. 1-12). This protein was different from trout P450 LMC1 or LMC2 and appeared to be the same P450 X1 ($M_r=52,000$) previously detected in some Mt. Shasta stock trout (Fig. 1-6 and Fig. 1-7). Although this protein comigrated with rat P450 2E1, we believe that this is not an ortholog of rat P450 2E1 since the band density did not correlated with PNP hydroxylase activity. Instead, we feel that this is a new, previously uncharacterized P450 isozyme, since we have detected this protein occasionally in other trout liver microsomal samples probed with anti-trout LMC2 IgG in Western blots (Fig. 1-10). The cross-reactivity between this protein and anti-rat P450 2E1 antibodies in the outbred trout liver microsomes was much less (only one-fifth as intense) than that found in albino and inbred trout.

Sex differences in P450 LMC2 in liver and kidney microsomes of mature rainbow trout have been reported (Williams et al., 1986). We confirmed their result that sexually mature trout displayed remarkably higher levels of P450 LMC2 in the liver than did sexually mature females (Fig. 1-13). However, our data revealed no difference in PNP hydroxylase activity between male and female trout liver microsomes (Fig. 1-14). These data suggest that P450 LMC2 is not a major contributor for NDMA activation in rainbow trout.

Williams et al.(1986) also demonstrated that the concentration of P450 LMC2 was considerably higher in male kidney microsomes than in male liver microsomes. This result was confirmed in our studies. Interestingly, both PNP hydroxylase activity and the intensity of the protein band

which comigrated with LMC2 and detected by anti-rat P450 2E1 were lower in male kidney than in male liver (data not shown). This implies that the major band comigrating with P450 LMC2 detected in almost all rainbow trout microsomal samples upon Western blotting with anti-rat P450 2E1 was not only P450 LMC2 but was a mixture of P450 LMC2 and the new P450 isozyme which has the same molecular weight ($M_r = 54,000$). We designated this new enzyme as P450 X2.

DISCUSSION

The dose of imidazole routinely used to induce P450 2E1 in mammals is 200 to 300 mg/kg body weight (Koop and Coon, 1984). However, these doses definitely could not be applied to the teleost system and only trout treated with 50 mg/kg survived the exposure. This latter dose, however, failed to induce the enzyme activity associated with P450 2E1. Imidazole is an inducer of P450 2E1 in rabbit but not in rat (Song et al., 1986). Due to such species differences of induction, the possibility of induction by imidazole in other fish species is still an open question.

Kaplan et al. (1991) demonstrated that the mRNA level of P450 pj, a P450 2E1 ortholog, in the fish Poecillio was increased after 24-hr treatment with 0.3 % ethanol, and decreased with the higher concentration of ethanol and longer exposure time. We chose, therefore, to use 0.25 % and 0.5 % ethanol to expose the rainbow trout in preliminary studies. PNP hydroxylase activity was very low in trout and was unchanged by ethanol exposure. We observed in Western blots two proteins that cross-reacted with anti-rat P450 2E1 antibodies. Since there was an observable increase in band intensity in the 0.5 % ethanol exposed trout liver microsomes as compared to the 0.25 % group, the time course response of the trout was, therefore, studied using 0.5 % ethanol.

There were no ideal ways to prepare approximately 30 gallons of 0.5 % ethanol solution. Thus the actual ethanol concentration which the fish exposed were somewhat different in each group. When the true ethanol concentrations were measured by using the enzyme kit, the 16 hour treatment solution was found to be higher (0.86 %), therefore, the high ethanol concentration and long time exposure may explain why the total cytochrome P450 content was highest in 16 hr-treatment female group. Since it was impossible to identify the sex until the fish were sacrificed, unfortunately, only one male fish was in the 16 hr-treatment group, we could not, therefore, make any conclusion with regard to enzyme concentration versus sex of the animals.

Glycerol can be metabolized by P450 2E1 in the rat, although it is not a direct substrate by P450 but is a substrate for an oxidant derived from interaction of iron with H_2O_2 generated by cytochrome P450 (Clejan and Cederbaum, 1991 and 1992). In isolated rat microsomes, glycerol inhibited pyrazole oxidation (a measure of P450 2E1 activity) by approximately 50 % when more than 150 mM glycerol was in the reconstituted system (Clejan and Cederbaum, 1990). When we added 200 μ l of 10 mg/ml microsome to 1 ml reaction mixture in our normal assay, the resulting glycerol concentration is 540 mM. There is a possibility, therefore, that glycerol could inhibit the enzyme. However, freshly prepared, glycerol-free microsomes did not show any increased PNP hydroxylase activity. Thus the glycerol effect on the enzyme activity might not be too important in the rainbow trout.

In rats, P450 2E1 was found to be a female predominant enzyme and the difference between the sexes was attributed to the male growth hormone secretion pattern which suppresses the expression of P450 2E1 (Waxman et al., 1989). However, Yamazoe et al. (1989) found that in rats P450 2E1 was male predominant. In contrast, Thummel and Schenkman (1990) suggested that there was no significant difference between the constitutive level

of P450 2E1 in male and female rats.

The PNP activity of trout was very low when 0.5 mg microsomes were incubated. We, therefore, increased the microsomal protein concentration to 2 mg in the 1 ml incubation mixture. Microsomes from control male trout showed slightly higher PNP hydroxylase activity than did the female microsomes (Fig. 1-3 and 1-4) but enzyme activities were much lower than those seen with untreated rat microsomes (Koop et al., 1989). However, only female trout hepatic microsome showed a slight but significant increase in PNP hydroxylase activity with time of ethanol exposure (Fig. 1-4). We hypothesized female trout were more sensitive to ethanol treatment than male trout. The PNP hydroxylase activity was also slightly higher in sexually mature males than in females.

Sheep anti-rabbit P450 2E1 antiserum has been used as a probe in Western blots in preliminary studies. Only one protein band which comigrated with P450 LMC1 was detected in trout liver microsomes by this antiserum. However, there was too much nonspecific binding that obscured the autoradiography. Accordingly, anti-rat P450 2E1 IgG was used for the subsequent screening immunoblot analysis. By contrast, the latter antibodies detected two proteins which comigrated with P450 LMC1 and LMC2. In some samples a weak third band (we designated it P450 X1) was detected migrating between the P450 LMC1 and LMC2 bands ($M_r = 52,000$). This latter band comigrated with rat P450 2E1. However, no increase in the concentration of any of the bands was found following ethanol pretreatment. Since the anti-rat P450 2E1 antibody recognized purified trout P450 LMC1 and LMC2, we concluded that the two cross-reacting proteins were detected in the microsomal samples probably P450 LMC1 and LMC2. When rabbit anti-trout P450 LMC1 and LMC2 was used to immunoquantitate P450 LMC1 and LMC2, no evidence of induction of these P450s was noted. Ronis et al. (1989) did not find any cross-reacting protein band after probing with rabbit anti-rat P450 2E1 IgG both in sea birds and fish

including shark, hagfish and perch. However, a single band reacting with anti-rat P450 2E1 was seen in the fish Poeciliopsis by Kaplan et al.(1991). Furthermore, our laboratory has shown that there was no cross-reacting band in hepatic microsomes isolated from Japanese quail upon blotting with anti-rat P450 2E1. We believe this is the first report that a polyclonal antibody raised against rat cytochrome P450 2E1 has strong cross-reactivity with more than two protein bands in the teleost. These findings suggest that there must be some reasonably close structural homology between rat P450 2E1 and trout P450 LMC1 and LMC2. Our laboratory has recently shown that trout P450 LMC2 belongs to a new subfamily (2K1) and that this isozyme is a member of the P450 2 family (Buhler et al., 1992).

According to these data, the two bands recognized by rabbit anti-rat P450 2E1 IgG in the present research were most likely P450 LMC1 and LMC2. If the PNP hydroxylase activity had been sufficiently high, antibody inhibition experiments might have been helpful to further characterize these proteins. However, Wrighton et al.(1991) have demonstrated that even monoclonal antibodies recognizing different epitopes on rat cytochrome P450 II B1 can react with human P450 2E1. It is thus possible for cross-reactions to occur between fairly dissimilar P450s which makes it even more difficult to draw conclusions from the results of the present studies. While female trout showed a small increase in PNP hydroxylase activity with ethanol exposure (Fig. 1-4), no corresponding increases in protein bands cross-reacting with anti-rat P450 2E1, were observed. We can not, therefore, establish with certainty that an ortholog of cytochrome P450 2E1 is present in rainbow trout liver. Although Kaplan et al.(1991) showed a single band in Poeciliopsis recognized by anti-rat P450 2E1 antibody, these researchers did not demonstrate a correlation between enzyme activity and protein concentrations as determined by immunoquantitation. In our studies, due to some

unknown reasons, the intensity of cross-reaction between the anti-rat P450 2E1 antibodies and the two protein bands comigrating P450 LMC1 and LMC2 was changed when repeat experiments were done using frozen microsomal preparations. Further investigations are necessary to determine the source of this variability. One possible explanation could be a partial denaturation of one of the trout P450s upon freezing with resulting alteration in the three-dimensional structure so that previously hidden epitopes become available to the antibody with resulting increases in cross-reactivity.

Sexually mature male and female trout liver microsomes were isolated and examined for P450 LMC2 content. Our results revealed that as in the previous report (Williams et al., 1986), expression of P450 LMC2 was higher in male than in female trout. In addition, PNP hydroxylase activity was slightly higher in the males than in females. However, there was no consistent relationship of the P450 LMC2 level and enzyme activity. Consequently, we concluded that since little PNP hydroxylase activity is associated with P450 LMC2, this isozyme is probably not responsible for the NDMA activation in trout. Some evidence for nitrosamine activation by endogenous P450s other than P450 2E1 has been reported for cultural human B-lymphoblastoid cells (Crespi et al., 1991). Williams et al. (1986) also demonstrated that the level of P450 LMC2 was higher in sexually mature male trout kidney than that in liver, and that P450 LMC2 was barely detectable in female trout kidney. We confirmed these researchers' results with Western blots employing rabbit anti-trout P450 LMC2 antibodies. PNP hydroxylase activity was slightly lower in kidney microsomes than in liver microsomes. When we probed the mature trout kidney microsomes with anti-rat P450 2E1 antibodies in Western blots, the intensity of the cross-reacting protein band which we had thought to be P450 LMC2 was less in mature male kidney than that found in mature male liver. These results suggested that the anti-rat P450 2E1

antibodies are recognizing an additional P450 isozyme (we designated it P450 X2) which comigrates with the P450 LMC2. The anti-rat P450 2E1 antibodies, therefore, are cross-reacting with both P450 LMC2 and this new P450 isozyme.

Kaplan et al.(1991) demonstrated there were strain difference in the NDEA deethylase activity in the viviparous fish Poecillio. Pedersen et al.(1975) reported that there was much variation in hepatic aryl hydrocarbon hydroxylase activity among rainbow trout strains and individuals within some stains. Our laboratory has shown the expression of P450 LMC1 and P450 1A1 were lowest in the albino trout. We, therefore, determined if PNP hydroxylase activity was higher in other trout strains- albino, inbred and outbred. This enzyme activity in inbred trout was found to be only two-fold that of outbred and albino trout. The 52,000 MW protein (P450 X1) which comigrated with rat P450 2E1 was detected in high concentration by rabbit anti-rat P450 2E1 antibodies in albino and inbred trout liver microsomes. However, no correlation was found between the PNP hydroxylase activity and immunoreactivity in these strains indicated this new isozyme is likely not responsible for the PNP hydroxylase activity in the trout.

In conclusion, slight induction of PNP hydroxylase activity by 0.5 % ethanol with exposure time was seen in female liver microsomes and the 24 hr-treated group demonstrated the significant difference from control fish. There were four proteins, presumably cytochrome P450 isozymes LMC1, LMC2, X1 ($M_r = 52,000$) which migrate between P450 LMC1 and LMC2, and X2 which comigrates with P450 LMC2 ($M_r = 54,000$), all of which cross-react with anti-rat P450 2E1 antibodies. If there was an ortholog of rat P450 2E1 in trout, the concentration of the isozyme might be too low to be detected in Western blots, since the related enzyme activity is not very high. The relative intensity of the three observable protein bands in Western blots of liver microsomes changed

when we repeated the experiments using stored microsomes. This phenomenon has not been found previously and the mechanism for these changes remains to be uncovered.

ACKNOWLEDGMENTS

Thanks to Dr. Denis R. Koop for providing sheep anti-rabbit cytochrome P450 2E1 IgG.

Table 1-1. Ethanol concentration before and after the exposure

sample collection	Time of exposure			
	0 hr	8 hr	16 hr	24 hr
before exposure	N.D.	0.6%	0.86%	0.57%
after exposure	N.D.	0.6%	0.59%	0.51%

Each value is an average of duplicate samples. N. D. represents no detectable.

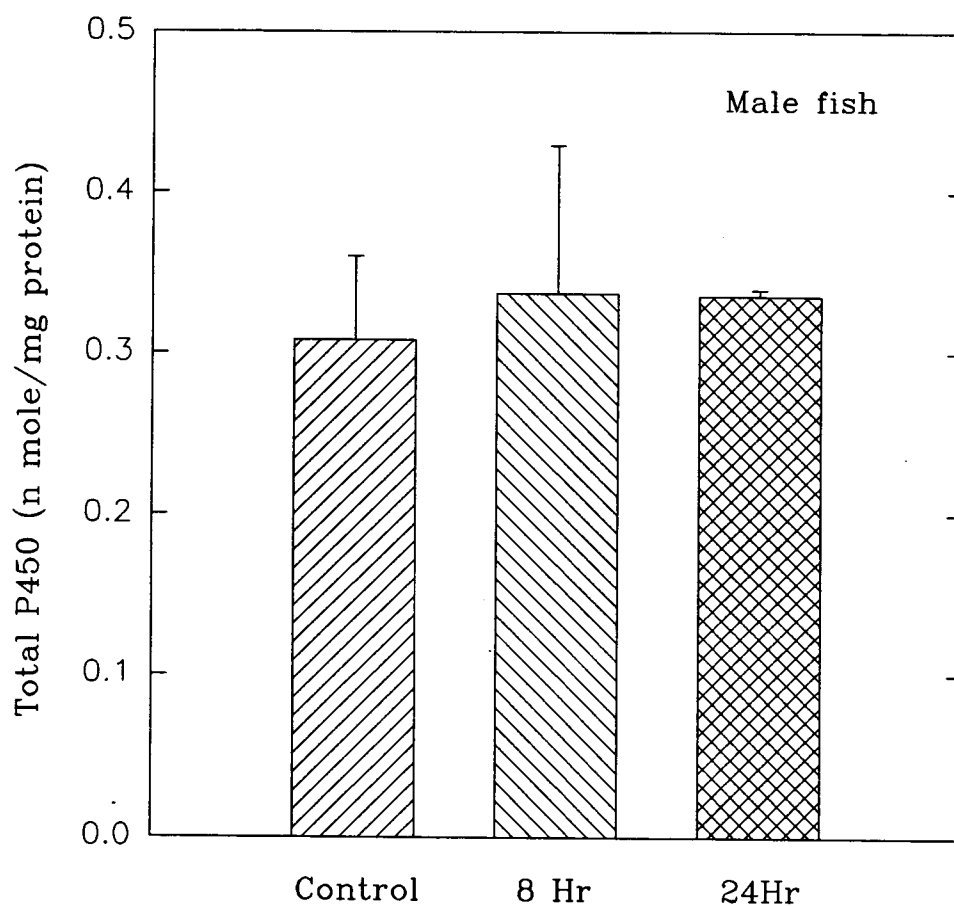


Fig. 1-1. Total cytochrome P-450 content in male hepatic microsomes. X axis represents time of exposure in 0.5% ethanol. All values are given as the mean \pm SD of three to four animals.

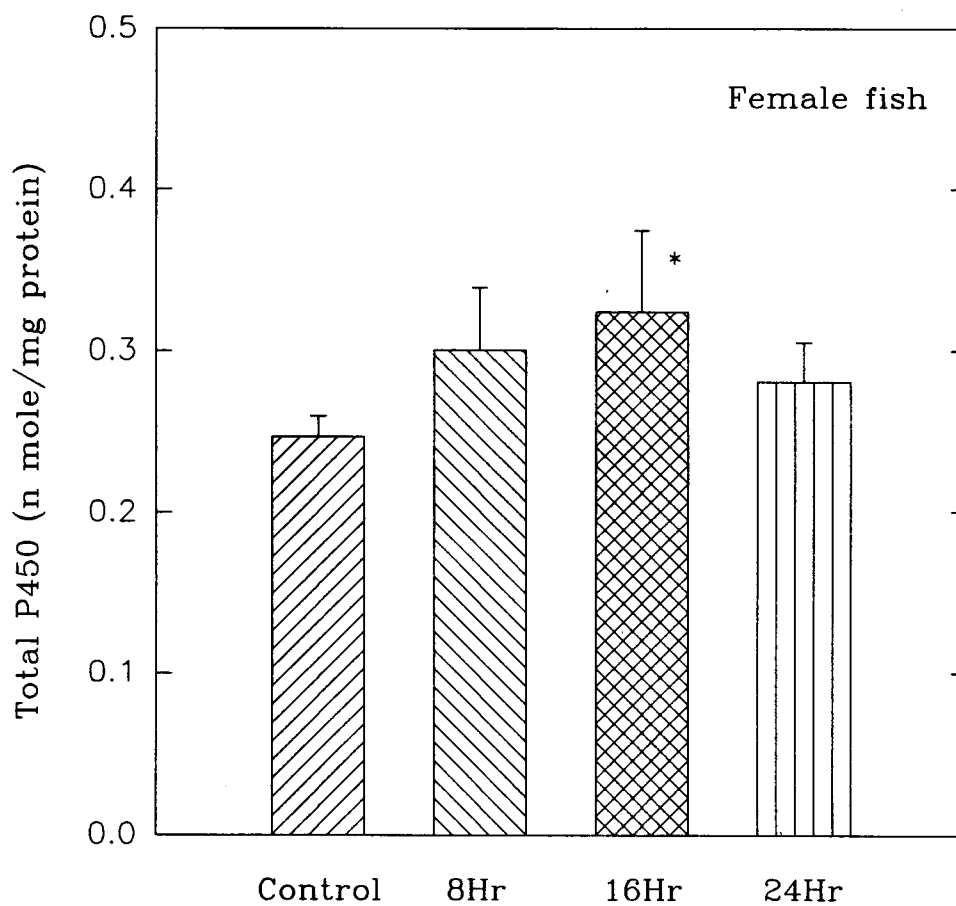


Fig. 1-2. Total cytochrome P-450 content in female hepatic microsomes. X axis represents time of exposure in 0.5% ethanol. All values are given as the mean \pm SD of two to four animals. *, $p < 0.05$.

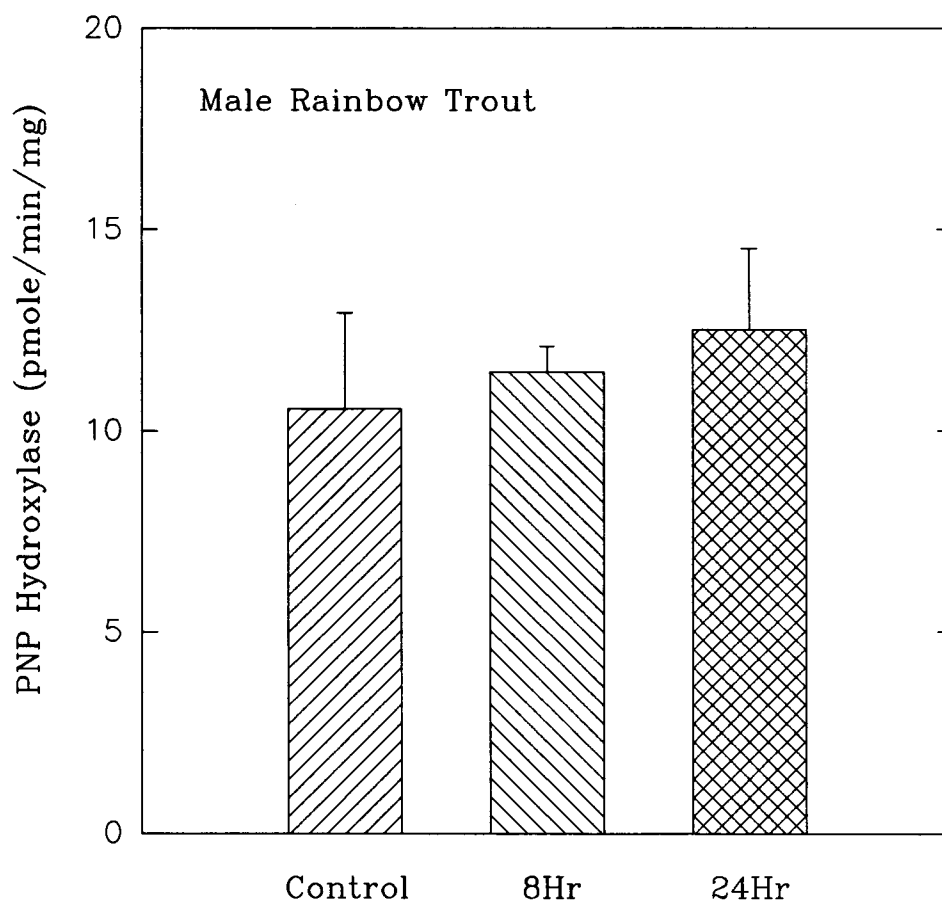


Fig. 1-3. p-Nitrophenol hydroxylase activity in male hepatic microsomes. The data shown are mean \pm SD of three to five animals performed in duplicate.

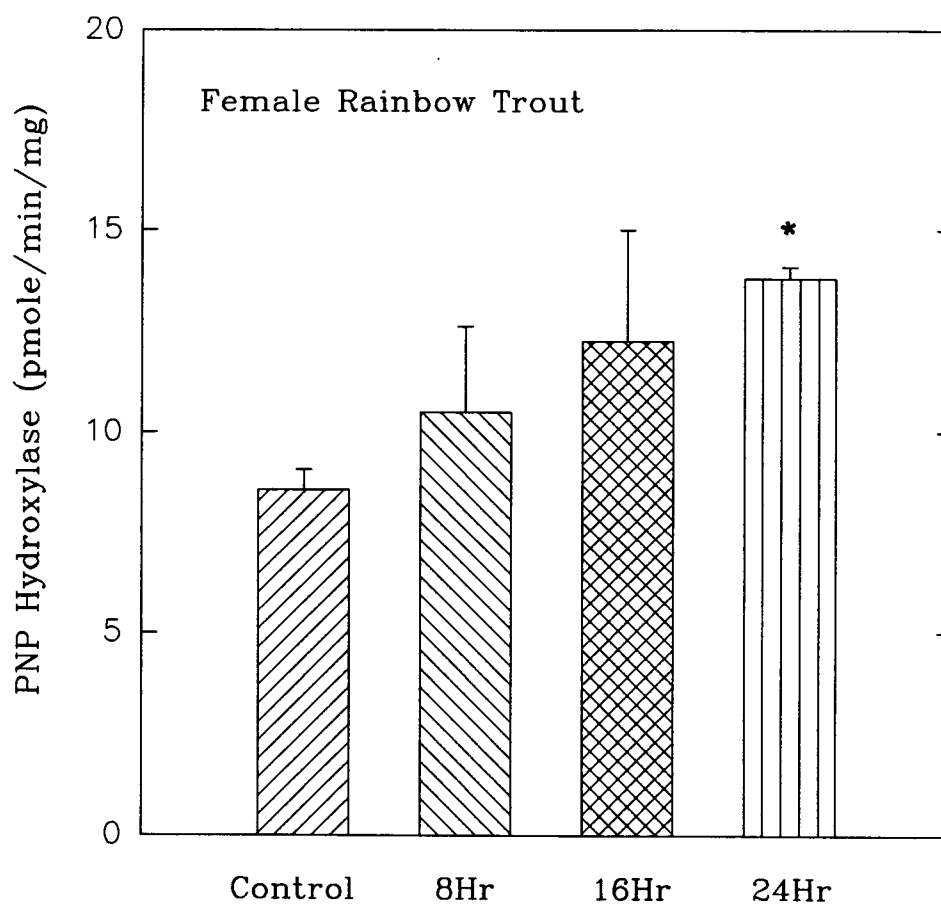


Fig. 1-4. p-Nitrophenol hydroxylase activity in female hepatic microsome. The data shown are mean \pm SD of two to four animals performed in duplicate. *, $p < 0.05$.

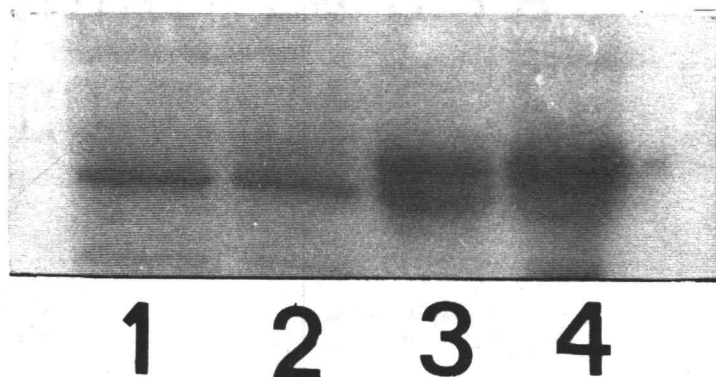


Fig. 1-5. Western blot analysis of trout and rat liver microsomal protein using sheep anti-rabbit P450 2E1 antibody as a probe. Lane 1, control female trout; Lane 2, 0.5 % ethanol 16 hr-treated female trout; Lane 3, control rat; Lane 4, pyridine-treated rat. Trout microsomes were 40 μ g per lane, while rat microsomes were 20 μ g per lane.

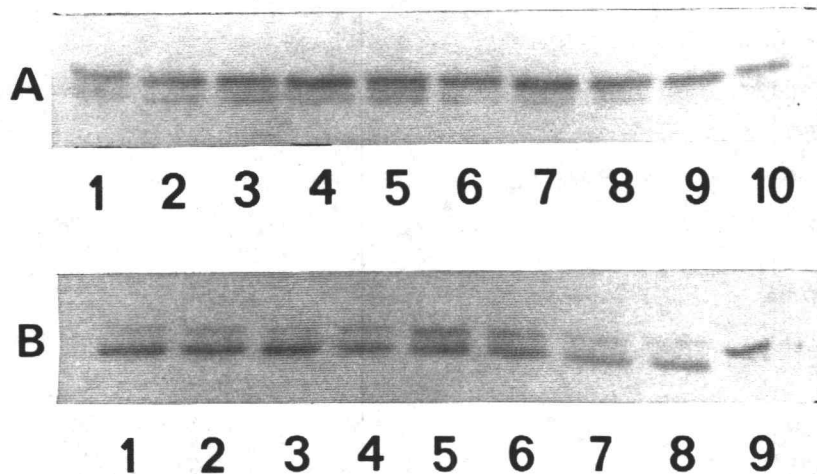


Fig. 1-6. Western blot analysis of liver microsomal protein isolated from control and ethanol-treated female trout using rabbit anti-rat P450 2E1 antibody as a probe. (A). Freshly prepared microsome. Lane 1 to 3, control ; lane 4 and 5, 8 hr exposure; lane 6 to 8, 16 hr exposure; 9 and 10, 24 hr exposure. (B). microsome after being stored. Lane 1 to 3, control; lane 4 to 8, 16 hr exposure; lane 9, control rat microsome. Lane 9 of blot (B) was loaded with 10 μ g protein, others were 40 μ g.

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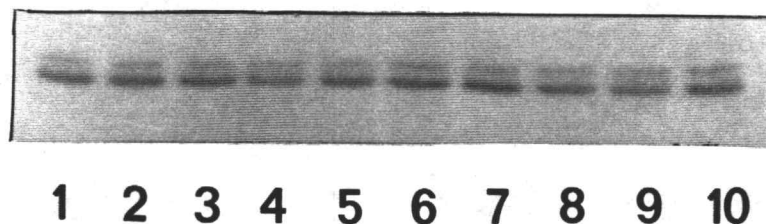


Fig. 1-7. Western blot analysis of liver microsomal protein isolated from control and ethanol-treated male trout using rabbit anti-rat P450 2E1 antibody as a probe. Lane 1 to 3, control; lane 4 to 7, 8 hr exposure; lane 8 to 10, 24 hr exposure. Each lane was loaded with 40 μ g protein.

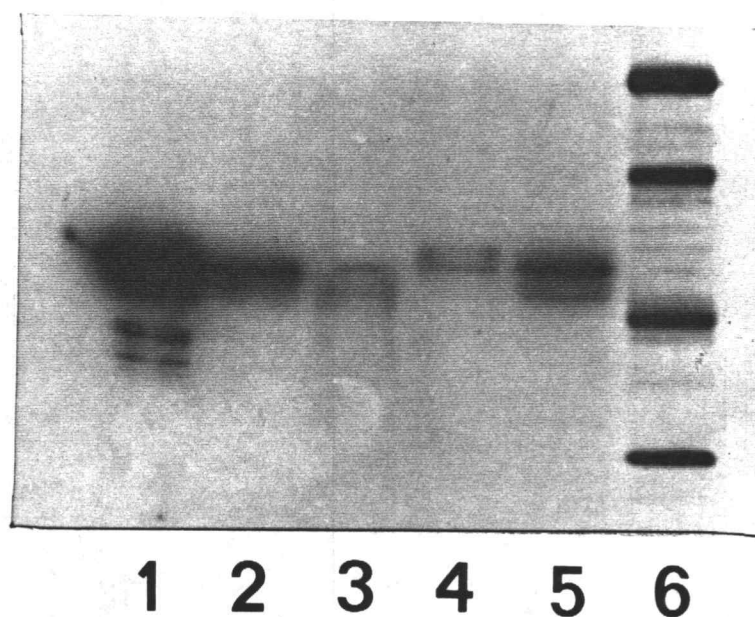


Fig. 1-8. Western blot analysis of trout cytochrome P450 LMC1 and LMC2 using rabbit anti-rat P450 2E1 antibody as a probe. Lane 1, pyridine-treated rat microsomes, 20 μ g; lane 2, control rat microsomes, 20 μ g; lane 3, 0.5 pmole LMC1; lane 4, 0.25 pmole LMC2; lane 5, 16 hr ethanol exposure trout microsomes, 40 μ g. lane 6, 14 C-labeled molecular standard. From top to bottom, were phosphorylase B (M_r = 97,400); bovine serum albumin (M_r = 68,000); ovalbumin (M_r = 43,000); carbonic anhydrase (M_r = 29,000).

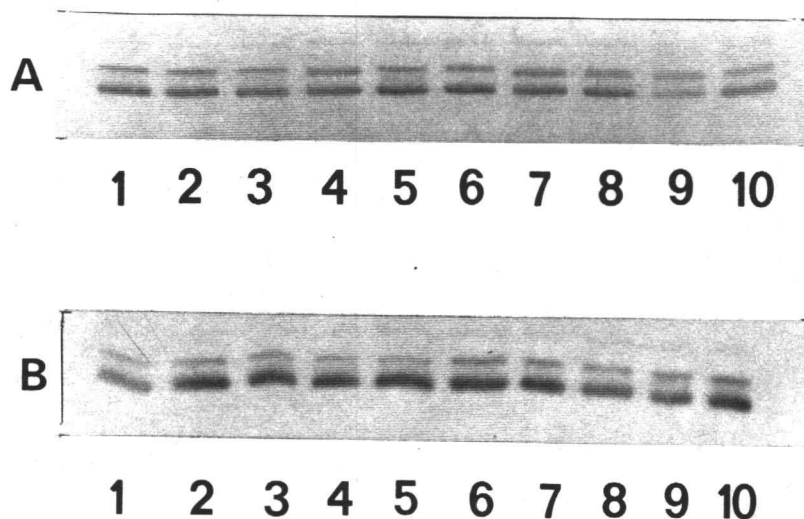


Fig. 1-9. Western blot analysis of liver microsomal protein isolated from control and ethanol-treated trout using rabbit anti-trout P450 LMC1 antibody as a probe. (A). female trout. Lane 1 to 3, control ; lane 4 and 5, 8 hr exposure; lane 6 to 8, 16 hr exposure; lane 9 and 10, 24 hr exposure. (B). male trout. Lane 1 to 3, control; lane 4 to 7, 8 hr exposure; lane 8 to 10, 24 hr exposure. Each lane was loaded with 20 μ g protein. There were two major bands, the upper one was LMC2, the lower one was LMC1.

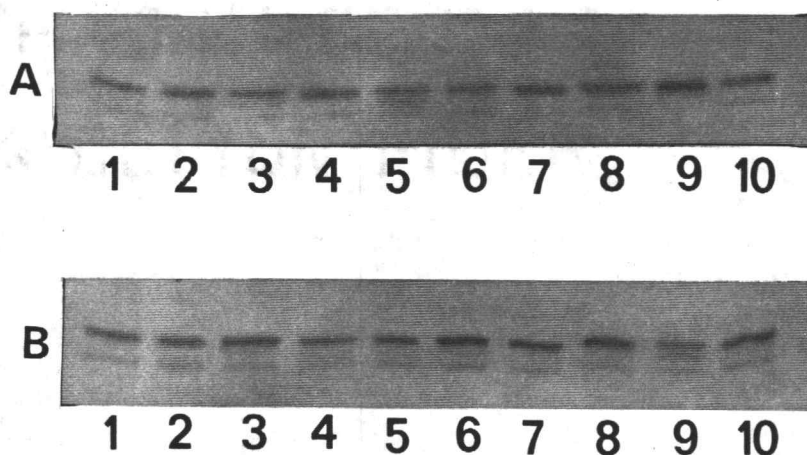


Fig. 1-10. Western blot analysis of liver microsomal protein isolated from control and ethanol-treated trout using rabbit anti-trout P450 LMC2 antibody as a probe. (A). female trout. Lane 1 to 3, control ; lane 4 and 5, 8 hr exposure; lane 6 to 8, 16 hr exposure; lane 9 and 10, 24 hr exposure. (B). male trout. Lane 1 to 3, control; lane 4 to 7, 8 hr exposure; lane 8 to 10, 24 hr exposure. Each lane was loaded with 20 μ g protein. The upper band was LMC2, the middle weak band was possible an unknown P450 isozyme (P450 X1), while the lower one was LMC1.

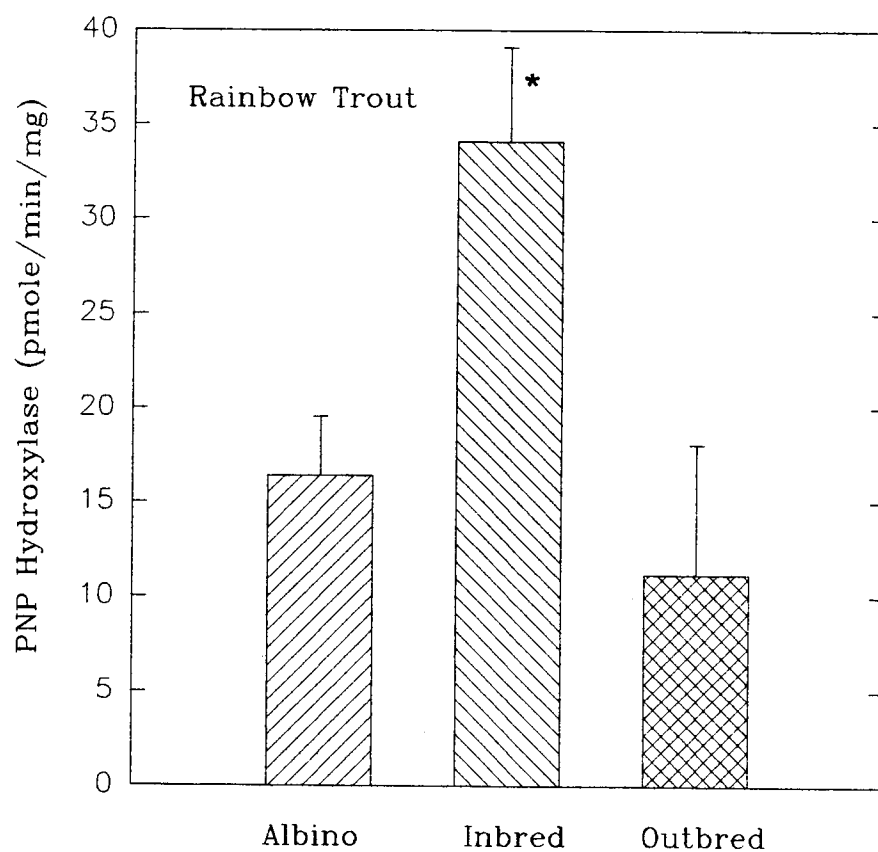


Fig. 1-11. p-Nitrophenol hydroxylase activity in albino, inbred and outbred trout hepatic microsomes. The data shown are mean \pm SD of three to four animals performed in duplicate. *, $p < 0.05$

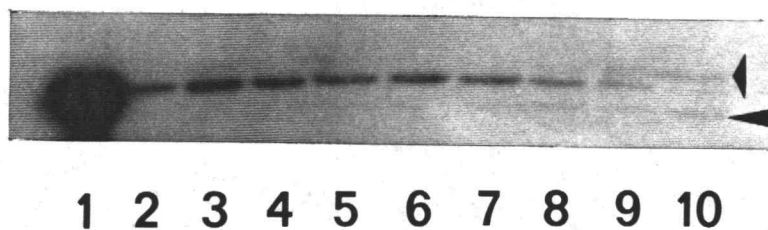


Fig. 1-12. Western blot analysis of hepatic microsomes from different strains of trout using rabbit anti-rat P450 2E1 antibody as a probe. Lane 1, pyridine-treated rat; Lane 2 to 4, albino trout; Lane 5 to 7, inbred trout; Lane 8 to 10, outbred trout. Forty μg of protein was loaded in each lane except 10 μg for rat microsomes. There were three bands on the blot. The big arrow and small arrow pointed out the position of LMC1 and LMC2 respectively. The major band (P450 X1) in albino and inbred trout comigrated with rat P450 2E1.



Fig. 1-13. Western blot analysis of liver microsomes isolated from sexually mature male and female using rabbit anti-trout P450 LMC2 antibody as a probe. Lane 1 to 3, male trout; Lane 4 to 6, female trout; Lane 7, purified P450 LMC2 (0.05 nmole). There was 20 μ g microsomal proteins in lane 1 to 6.

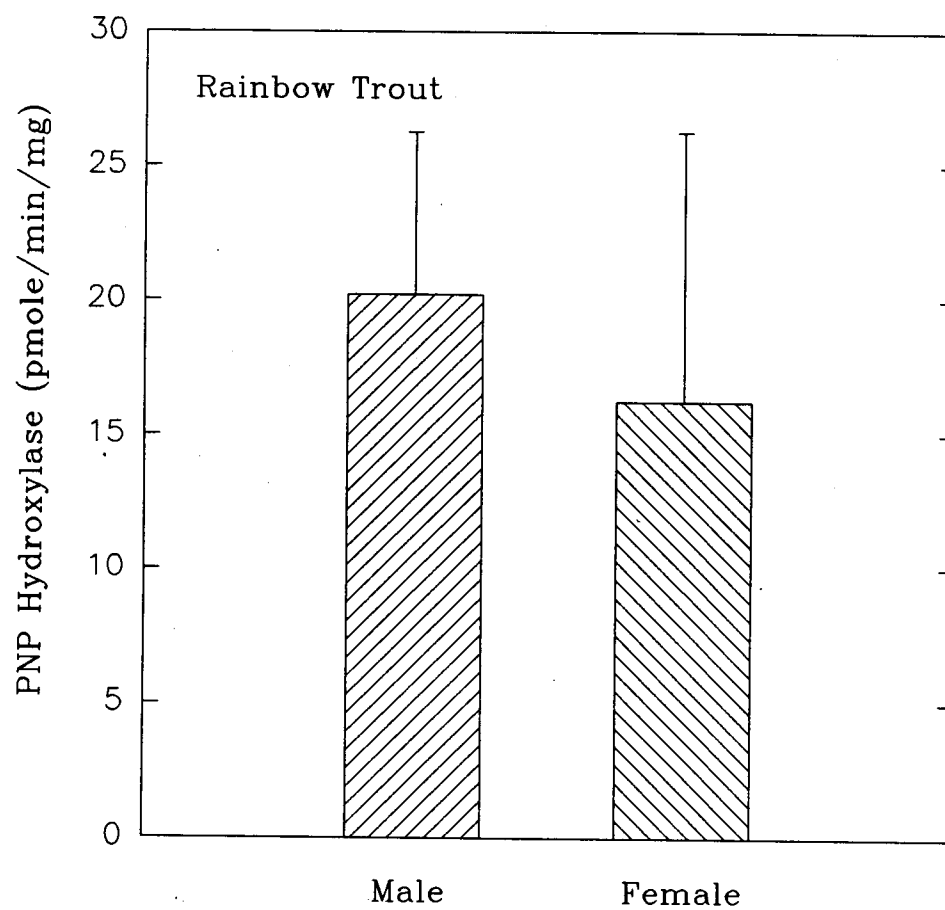


Fig. 1-14. p-Nitrophenol hydroxylase activity in sexually mature male and female trout hepatic microsomes. The data shown are mean \pm SD of three animals performed in duplicate. *, $p < 0.05$

Chapter 2

Expression of Glutathione S-transferases in Preneoplastic and Neoplastic Lesions of Rainbow Trout During Aflatoxin B₁-induced Hepatocarcinogenesis

ABSTRACT

Rainbow trout (*Oncorhynchus mykiss*) and rats have been shown to be very susceptible to aflatoxin B₁ carcinogenicity. In the rat, the expression of xenobiotic metabolizing enzymes (phase I and phase II) are changed in the preneoplastic foci. The glutathione S-transferase Pi (GST- π) family is highly induced in the altered hepatocellular foci and neoplasms. Therefore, GST- π has become a sensitive biomarker, replacing γ -glutamyltranspeptidase, for the diagnosis of early carcinogenesis. In this study, cytosolic GST was purified from rainbow trout liver and rabbit polyclonal antibodies then prepared against the purified trout enzyme. The N-terminal amino acids sequence of trout GST was determined and based on polyacrylamide gel electrophoresis (PAGE) evidence of only a single subunit and the N-terminal sequence very similar to that of published GST- π sequence, it was concluded that the trout GST belonged to π family. In order to determine if trout GST- π is a biomarker of carcinogenesis of AFB₁-induced tumors in the rainbow trout, we exposed the trout fry in 0.5 ppm AFB₁ for 30 min, and groups of fish were then killed every two weeks after exposure. Hepatic GST activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate in control and AFB₁-treated trout liver. Total GST- π concentrations in liver cytosol also were determined by Western blotting using the rabbit antibodies raised against the trout GST- π . Histological sections from untreated and treated fish were probed with rabbit anti-trout GST by using the avidin-biotin-complex (ABC) technique. There was no difference in cytosolic and microsomal

GST activity between control and treated fish. Moreover, no differences in cytosolic GST activity was observed between the tumor and non-tumor sections of the liver. However, the microsomal GST activity of tumor tissue was only one-half that of control fish and the non-tumor regions of treated fish. GST- π were expressed in normal tissue, and most of the cytoplasm and some nuclei in parenchymal cells were intensely immunostained. During carcinogenesis, immunohistochemical techniques showed that necrotic cells were strongly stained, but no evidence of GST- π expression was seen in regenerating cells. The preneoplastic foci and liver neoplasm were deficient in immunoreactive GST- π . By contrast, the altered hepatic foci and liver carcinoma from AFB₁-treated rats showed a strongly positive staining when using rabbit anti-trout GST- π IgG as a probe. No immunostaining was detected in the tissue surrounding the neoplasm in rat tissue. The GSTs of trout were detected in Western blots by rabbit anti-human placenta GST- π IgG. Unlike chemical carcinogenesis in the rat, GST- π in rainbow trout is expressed in normal liver tissue but is deficient in preneoplastic foci and adenocarcinoma. The loss of GST- π in trout preneoplastic hepatocytes may make these cells more susceptible to either endogenous or exogenous compounds which are normally detoxified by this GST isozyme.

INTRODUCTION

The glutathione S-transferases (EC 2. 5. 1. 18) are a family of multi-functional detoxification enzymes (Jakoby, 1978). They act, in the main, by conjugating reduced glutathione (GSH) with a range of electrophilic compounds of both endogenous and exogenous origins, giving products that may be excreted either directly or after further transformation (Boyland and Chasseaud, 1969; Meister, 1981). Glutathione S-transferase (GST) activity is distributed in the cytosol, microsomes, and nuclei (Morgenstern et al., 1982;

Tirmenstein and Reed, 1989).

Cytosolic GST is either a homodimer or heterodimer. At least 10 different subunits have been identified in the rat. GSTs are multigene families designated α family (subunits 1, 2, 8, and 10), μ family (subunits 3, 4, 6, and 9) π family (subunit 7 only). According to the mobility of the subunits on the SDS-PAGE, subunit 1, 2, 3, 4, 6 and 7 are named as Ya, Yc, Yb¹, Yb², Yn and Yf(Yp), respectively (Ketterer et al., 1988)

The GSTs are widely distributed in the animal kingdom and are found in many animal tissues. GST activity has been detected in gills, kidney, intestinal caeca and liver of rainbow trout (Bauermeister et al., 1983; Nimmo, 1985 and 1986; Nimmo and Spalding, 1985). Hepatic GSTs have been purified from rainbow trout and proved to be dimeric proteins (Rammage and Nimmo, 1983 and 1984).

The potent hepatotoxin and hepatocarcinogen, aflatoxin B₁ (AFB₁) is metabolically activated in the liver to form a reactive electrophile believed to be a labile epoxide (Essigmann et al., 1977; Swenson et al., 1977). This metabolite can bind to cellular macromolecules including DNA which results in neoplasia (Essigmann et al., 1982). Alternatively, AFB₁ is capable of GST-mediated conjugation with GSH (Lotlikar et al., 1984). Species vary greatly in their sensitivity to AFB₁ toxicity, which is in part dependent on their capabilities to activate the toxin, but also is related to their capacity to detoxify the reactive metabolite, by conjugation with GSH (O'Brien et al., 1983).

In the trout, the metabolites of AFB₁ are AFB₁-8,9-epoxide (which is believed to be capable of covalently binding to DNA and initiating cancer), aflatoxicol (AFL), aflatoxin M₁ (AFM₁), and aflatoxicol M₁ (AFL-M₁) (Loveland et al., 1983). In contrast to mammalian systems in which GSH conjugation of AFB₁-8,9-epoxide is a major detoxification pathway (Degen and Neumann, 1978), AFB₁ is poorly detoxified via GSH in the trout

(Valsta et al., 1988). Wallace (1989) suggested that rainbow trout and fathead minnows are more susceptible than rodents to xenobiotics that are eliminated principally by GSH-dependent metabolic detoxification because of limited availability of endogenous GSH in the fish.

Chemical hepatocarcinogenesis has been most extensively studied in rats. Initiation, promotion, and progression are thought to be the three stages of chemical carcinogenesis (Pitot and Sirica, 1980). During the promotion stage, groups of preneoplastic cells have been observed in many organs prior to appearance of malignant cancers. These preneoplastic cells not only have special structural features but also have unique biochemical patterns. Expression of xenobiotic-metabolizing enzymes, such as monooxygenases and GSTs, are commonly altered in these preneoplastic foci (also known as enzyme-altered foci) (Farber, 1984). Kitahara et al. (1983) found GST-A (YbYb) was increased markedly in rat liver preneoplastic lesions, and suggested form A could be a preneoplastic enzyme marker for chemical carcinogenesis. However, GST- π (purified from rat placenta) (GST-P) has been identified as a new more reliable marker enzyme for preneoplastic lesions in rat hepatocarcinogenesis (Kitahara et al., 1984; Tatematsu et al., 1985). GST- π was found to be localized in preneoplastic lesions of AFB₁-treated rat by immunohistochemistry, although this isozyme does not have significant GSH-conjugating activity towards AFB₁ metabolites (Neal et al., 1987). The aim of this project was to use immunohistochemical methodology to determine whether GST is a marker of preneoplastic foci in rainbow trout.

MATERIALS AND METHODS

A. Chemicals.

3,3'-Diaminobenzidine tetrahydrochloride (DAB), phenylhydrazine hydrochloride, poly-L-lysine, 1-chloro-2,4-

dinitrobenzene (CDNB), and Trizma base were purchased from Sigma Chemical Co. (St. Louis, MO); rabbit IgG vectastain ABC elite kit from Vector Laboratories (Burlingame, CA); rabbit anti-human placenta GST IgG from Crystal Chem (Chicago, IL) paraplast X-TRA tissue embedding media from Monoject Scientific (St. Louis, MO), and AFB₁ from Calbiochem (San Diego, CA). The chemicals and materials used in Western blotting and SDS-PAGE were purchased from Bio-Rad Laboratories (Richmond, CA) and [¹²⁵I]protein A were obtained from Amersham (Arlington Heights, IL). All other chemicals were reagent grade and commercially available.

B. Purification of GST and Preparation of Antibodies

β -Naphthoflavone-treated male trout liver cytosol was pooled and chromatographed on a column Sephadex G-75 followed by an affinity column containing S-hexylglutathione agarose, and chromatofocusing using column PBE94 (Kennish et al., 1992). GST activity in the fractions was measured using CDNB as substrate. Antibodies to GST of trout liver were prepared by inoculating New Zealand white rabbits with the purified trout GST. The IgG fraction of the sera was obtained by DEAE column chromatography (Kennish et al., 1992).

C. Analysis of N-terminal Amino Acids of GST.

SDS-polyacrylamide gels electrophoresis was carried out modified from the method of Laemmli (1970). Purified GST was applied to a 15 % gel. Electroblothing was carried out using Immunobilon-P (Millipore, Inc). The membrane was stained in 0.1 % Coomassie blue in 50 % methanol. The stained GST band was cut and sent to the Medical College of Wisconsin Protein/Nucleic Acid Shared Facility of the Cancer Center for analysis of the 15 amino acids of N-terminal sequence.

D. Treatment of Animals

Mt. Shasta strain rainbow trout (Oncorhynchus mykiss) were spawned at the Food Toxicology and Nutrition Laboratory, Oregon State University. Groups of 200 fry were randomly selected and reared in 4-ft-diameter fiberglass tanks and exposed to a 12 hr/12 hr light/dark cycle. Disease-free water was pumped from wells at a temperature of 12.5°C. Fry (two months after hatching) were separated into two groups. Control fish were untreated. One hundred treated fish were exposed to 0.5 ppm AFB₁ by immersion for 30 minutes and then transferred to carcinogen free water. Both groups of fish were fed a control diet (Sinnhuber et al., 1977b) until the time of sacrifice. Fish were randomly selected from each of the untreated (n=12) and AFB₁-treated (n=12) groups and killed every 2 weeks or 4 weeks after treatment. For immunohistochemistry, livers were fixed in 10 % neutral buffered formalin. For biochemical assay, six livers were pooled and homogenized in homogenization buffer.

E. Immunohistochemistry

Five-millimeter sections of the livers were fixed by gentle agitation for 3 hours at room temperature in neutral buffered formalin. Tissues were washed twice for 20 minutes each with 0.1 M phosphate buffer, pH 7.4, then placed in cold 0.1 M phosphate buffer, pH 7.4, which contained 10 % sucrose and held at 4°C overnight. The next morning tissues were embedded in paraplast embedding media and the paraffin blocks were stored at 4°C until sections were cut. Three- to four-micrometer sections were cut, placed on poly-L-lysine-pretreated glass slides, and stored at 4°C prior to immunohistochemical treatment.

An immunoperoxidase procedure (Hsu et al., 1981) was used that employed unlabeled primary antibody (diluted 1:800 in

0.01 M phosphate-buffered saline, overnight at 4°C) followed by biotinylated secondary antibody (60 minutes at room temperature), and then performed avidin and biotinylated horseradish peroxidase complex (60 minutes, room temperature) (Vectastain ABC elite kit). Endogenous peroxidases were inhibited by treatment of tissues with 0.1 % phenylhydrazine in Tris-buffered saline for 60 minutes. DAB was used as the chromogen, and Mayer's hematoxylin was used for the counterstain.

After staining with several dilutions of the primary antibody ranging from 1:200 to 1:1600, a working dilution of 1:800 was chosen to demonstrate specific staining with minimal background. Negative controls consisted of serial sections of the liver tissues treated in the same manner as the other tissues except that normal rabbit serum diluted 1:800 was substituted for the primary antibody. Positive controls consisted of renal tissue sections collected from same fish and AFB₁-treated-rat hepatoma tissue. Tissue sections were examined by conventional light microscopy.

F. Biochemical Assays

Pooled livers were homogenized in three time volume of 0.1 M Tris buffer, pH 7.5, containing 0.15 M potassium chloride and 1 mM EDTA, with eight up-and-down strokes in a motor-driven Potter-Elvehjem homogenizer, equipped with a Teflon pestle. Cytosol for the determination of GST was prepared by centrifuging the homogenate (100,000 g, 90 min, 4°C) and glycerol was added to 20 % of final concentration. Microsomal pellets were resuspended in 0.1 M potassium phosphate buffer, pH 7.25, containing 1 mM EDTA, 1mM DTT and 20 % glycerol.

The GST activity was determined according to the method of Habig et al.(1974). The reaction mixture contained the substrate GSH and CDNB at 1 mM in 1 ml 0.1 M potassium

phosphate buffer, pH 6.5; 0.02 ml appropriately diluted cytosol was added and the increase in absorbance at 340 nm monitored. Specific activity is expressed in units of enzyme activity/mg of protein, as measured by the Lowry (1951) method with bovine serum albumin as standard.

Cytosol and microsomes were used for the SDS-PAGE and Western blotting. SDS-PAGE was performed by the method of Laemmli (1970) and the procedure for Western blotting using [¹²⁵I]Protein A was according to Burnette (1981). Proteins separated by 15 % polyacrylamide slab gels were electrophoretically transferred (20 V, 30 min, 4°C) to nitrocellulose and immunostained with rabbit anti-trout GST IgG followed by [¹²⁵I]Protein A. Visualization was performed by autoradiography. Immunodetection was done by scanning the autoradiography on a densitometer.

RESULTS

There were three major peaks in the elution profile of GST from chromatofocusing using column PBE94. Fractions from each peak were pooled and applied to SDS-PAGE. The fractions from these three peaks gave one band of M_r 23,000, implying that these proteins are homodimers (Kennish et al., 1992). The 15 amino acids from N-terminal sequence of the rainbow trout GST are shown in Fig. 2-1. Over the region sequenced, trout GST has high homology with the pig π -class GST (Nishinaka, et al., 1991; Dirr et al., 1991), the rat GST- π (Okuda et al., 1987) and the GST isolated from bovine placenta (Schaeffer et al., 1988). This result is similar to the finding from Dominey et al. (1991) who observed that the major GST in salmon liver possessed about 65 % homology with rat and human π -class GST, and the subunits of GST isolated from sea trout, salmon, and rainbow trout have cross-reactivity with the rat π -class Yf subunit of GST. Among the 15 residues of the trout GST that were sequenced, only the 11th amino acid is different from

that of salmon GST- π .

There was uniform staining for GST- π within the cytoplasm of the parenchymal cells in normal hepatic tissue (Fig. 2-2). Nuclei of some parenchymal cells were stained but there was no typical pattern. Specific staining for GST- π was also observed along the epithelial surface of the gall bladder. In the trunk kidney of control and treated trout, anti-GST- π stained the cytoplasm and some nuclei of cells of proximal tubules (Fig. 2-3). The cytoplasm of interrenal cells in head kidney tissue sections from both groups showed intense, homogeneous immunostaining by anti-GST- π IgG. There appeared to be no difference in the staining characteristics of the kidney of both groups.

The amount of nonspecific background staining present in any section was nondetectable. No staining or faint staining was discernable on the negative control slides. Cytoplasm and some nuclei of parenchymal cells of rat hepatoma and preneoplastic foci from AFB₁-treated rat were strongly stained by rabbit anti-trout GST- π IgG (Fig. 2-4). Surprisingly, both cytoplasm and nuclei of basophilic foci from AFB₁-treated trout liver tissue gave no reaction with the GST- π antibodies, and moreover, no detectable staining of GST- π was observed in the rainbow trout preneoplastic nodules and cholangioma (Fig. 2-5 and 2-6). Necrotic cells were stained intensely, but regenerating cells were not stained at all on AFB₁-treated fish tissue sections. These unexpected results are contradictory to those found in rat hepatoma.

Both cytosolic and microsomal GST activity continuously increased with the age of the fish (Fig. 2-7). There was no difference of cytosolic GST activity between control and treated trout. There was also no difference in microsomal GST activity between control and AFB₁-treated rainbow trout (Fig. 2-8). However, microsomal GST activity of tumor tissue was about 50 % that of control fish and tumor-free liver from AFB₁-treated trout (Table 2-1).

Only one trout liver cytosolic protein co-migrating with purified GST- π and was detected by anti-GST- π IgG on the blots from both groups of fish (Fig. 2-9). We believe this protein was GST- π . The density of the protein band was then scanned but no significant difference of GST- π content was found between the untreated and tumor induced groups. We also probed the purified trout GST- π and the trout liver cytosol with rabbit anti-human GST- π on Western blots. There was strong cross-reaction between the trout GST- π and the latter antibodies (Fig. 2-10).

DISCUSSION

There is a well-recognized range of sensitivities among different species with respect to hepatotoxicity and carcinogenicity of AFB₁. Rainbow trout are extremely responsive to the carcinogenic activity of AFB₁ either at the dose of 1.0 ppb in the diet or after immersion at 0.5 ppm for 30 minutes (Sinnhuber et al., 1977a). Rats are also sensitive to AFB₁ and AFB₁-induced carcinogenesis has been studied extensively in this species. During the process of hepatocarcinogenesis, the enzyme-altered foci, also known as preneoplastic foci, are believed to be involved in the hepatocellular carcinoma formation (Pitot, 1990). The activity and protein content of GST-A (subunits 3 and 3') was increased markedly in rat preneoplastic hepatic lesions induced by diethylnitrosamine followed by administration of N-2-fluorenylacetamide (Kitahara et al., 1983). GST-B (subunits 1 and 2) and GST-C (subunits 3 and 4) were increased in all preneoplastic and neoplastic lesions in liver when rats were treated with nitrosamine (Buchmann et al., 1985). More recently, GST- π (placenta form) is believed to be the most accurate marker enzyme for detection of initiated cells during liver carcinogenesis (Tatematsu et al., 1988). Our purified

trout GST was classified as belonging to be π family (7-7 or Yf-Yf). It was hypothesized that the phenotype expression of GST in hepatoma from AFB₁-treated trout would be similar to that in rat. On the contrary, we could not find any staining of GST- π in altered hepatic foci in rainbow trout by the ABC method using anti-GST- π antibodies in rainbow trout. Kirby et al, (1990) also reported that in rainbow trout a majority of liver tumors (76 %) were deficient in immunoreactive GST whereas 10 % of the neoplasms had a similar content when compared with surrounding non-neoplastic liver. Stalker et al. (1991) have demonstrated that the major constitutive GST was not expressed in various liver lesions in white suckers captured from Lake Ontario. These researchers also suggested that the progression of liver neoplasia in fish involves loss of GST, a change that might be important in increasing susceptibility of preneoplastic populations of hepatocytes to DNA damage by other xenobiotics.

The anti-trout GST- π IgG did recognize the preneoplastic foci and neoplasms and did not stain the normal tissue in the rats which are treated with AFB₁. However, the nuclei of cells of altered hepatic foci from AFB₁-treated rat were stained by antibodies against trout GST- π . The positive staining of nuclei was also found by Power et al. (1987) when antibody against rat GST- π was used in immunohistochemistry. Sato et al. (1987) has demonstrated GST- π levels in rat normal tissues, including placenta, fetal and adult livers, are generally very low. The kidney (proximal and distal tubules), lung (bronchiolar epithelial cells) and pancreas (acinar cells) contain significant amounts of GST- π . Using the immunohistochemical staining we found GST- π was highly expressed in trout kidney (proximal tubules), head kidney (interrenal cells), gill (epithelial cells), and intestine (epithelial cells).

It has been shown that CDNB is also a substrate of microsomal GST in rodents (Morgenstern et al., 1984). With

CDNB as a substrate, there was no evidence of different cytosolic and microsomal GST activity between control and treated trout. We also found microsomal GST activity of trout tumor was one-half of that of tumor-free tissue from treated group and control group. A microsomal GST has been described from rat and mouse (Morgenstern et al., 1982, Anderson et al., 1988). In the rat, the microsomal GST possess characteristics that are clearly distinct from those of cytosolic GST including amino acid sequence, subunits Mr, enzymatic properties. The anti-trout cytosolic GST- π IgG cross-reacted with the isolated microsomal protein which co-migrated with trout cytosolic GST- π on the SDS-PAGE. Further studies are necessary to establish whether this microsomal protein is actually a GST- π .

The mechanism of regulation of GST expression in enzyme-altered foci and neoplastic tissue in rainbow trout liver is still unclear. Among mammals, the mouse is more resistant than the rat because mouse hepatic cytosol has GSTs that more effectively conjugates the AFB₁-epoxide with GSH than does rat hepatic cytosol (Degen and Neumann, 1981). Recently, Quinn et al. (1990) has shown that a constitutive GST isozyme (Ya Ya or 4-4) in mouse liver strongly protects DNA from AFB₁ metabolites. However, several types of xenobiotic-induced resistance in rat liver can be explained largely by an increase in total GSTs but not by the expression of GST isozymes with a greater DNA protective activity toward AFB₁ intermediates. Quinn et al. (1990) also demonstrated that various hepatic GSTs, including rat GST- π , trout total GSTs and human total GSTs, have a low DNA protective activity toward AFB₁ metabolites generated by rat hepatic microsomes.

AFB₁-GSH conjugation is a major determinant of AFB₁ carcinogenesis in rodent models and this relationship has also been suggested to apply to fish as well. Rainbow trout are known to be more susceptible to AFB₁ hepatic carcinogenesis than coho salmon. However, Valsta et al. (1988) found that

AFB₁-GSH conjugate was not detected in salmon bile but this conjugate was present in trout bile in amount representing less than 0.2 % of the administered dose 24 hours after injection of [³H]AFB₁. GST activity in control trout livers was much higher than that in the control salmon livers. Consequently, there must be a mechanism other than GST detoxification to explain the resistance of salmon to AFB₁ carcinogenesis. Further research is needed to establish the relationship between GST- π and chemical carcinogenesis in the rainbow trout.

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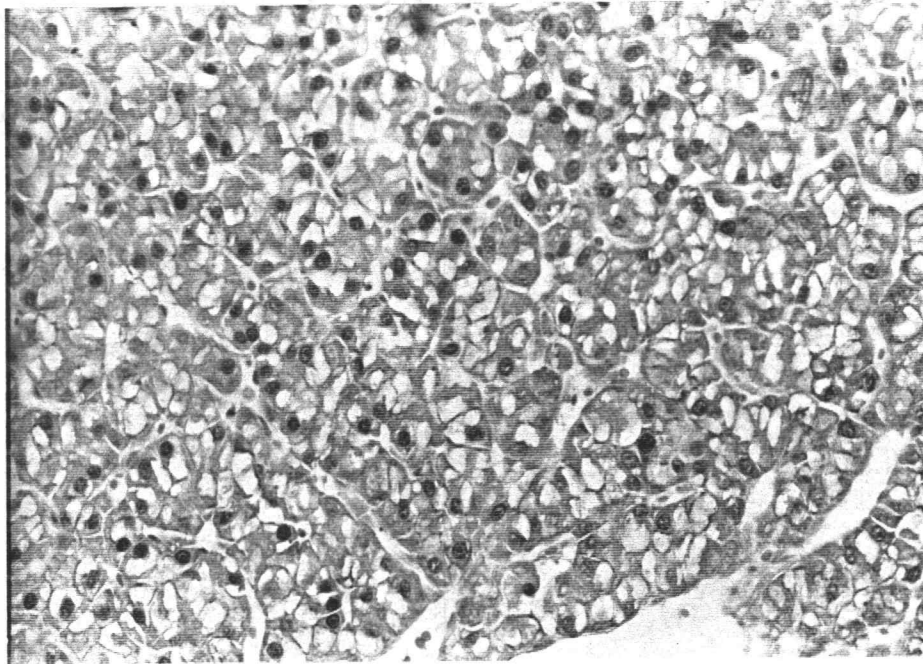


Fig. 2-2. Liver tissue from untreated rainbow trout stained for GST- π illustrating positive staining for parenchymal cells. Note the nuclei of some cells were strongly stained (X 340).

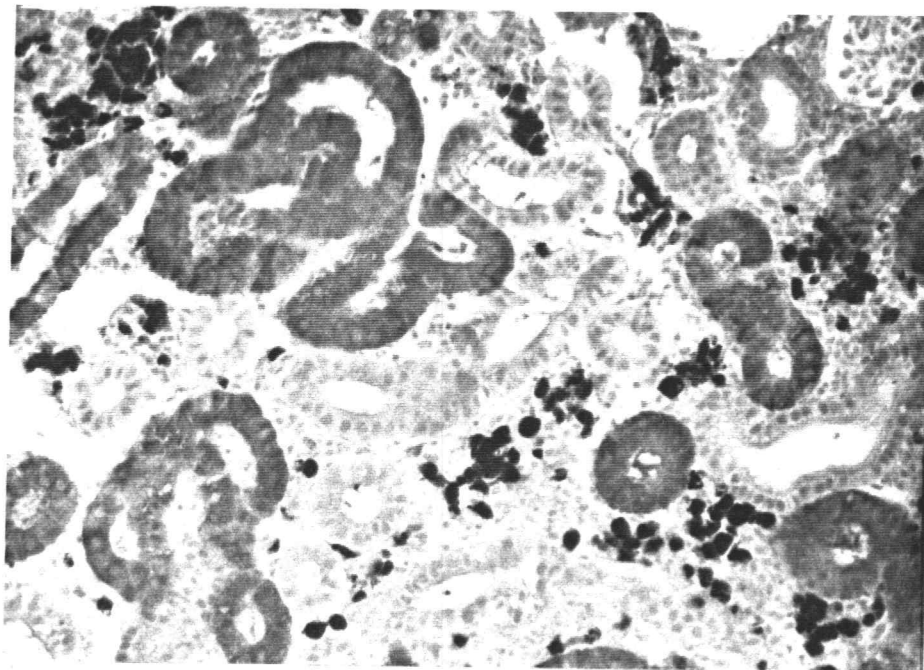


Fig. 2-3. Trout kidney tissue stained for GST- π illustrating cytoplasm of proximal tubules was strongly stained (X 340).

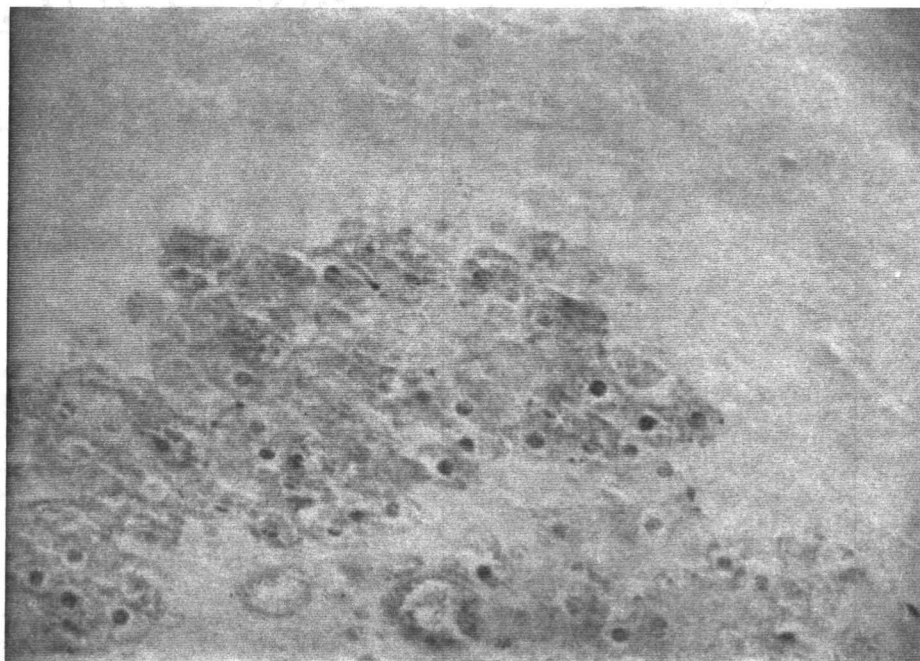


Fig. 2-4. Ppreneoplastic foci in AFB₁-treated rat liver stained for GST- π . Ppreneoplastic foci were stained positively (bottom) comparing with the negatively stained surrounding cells (top) using anti-trout GST- π antibody (X 340).

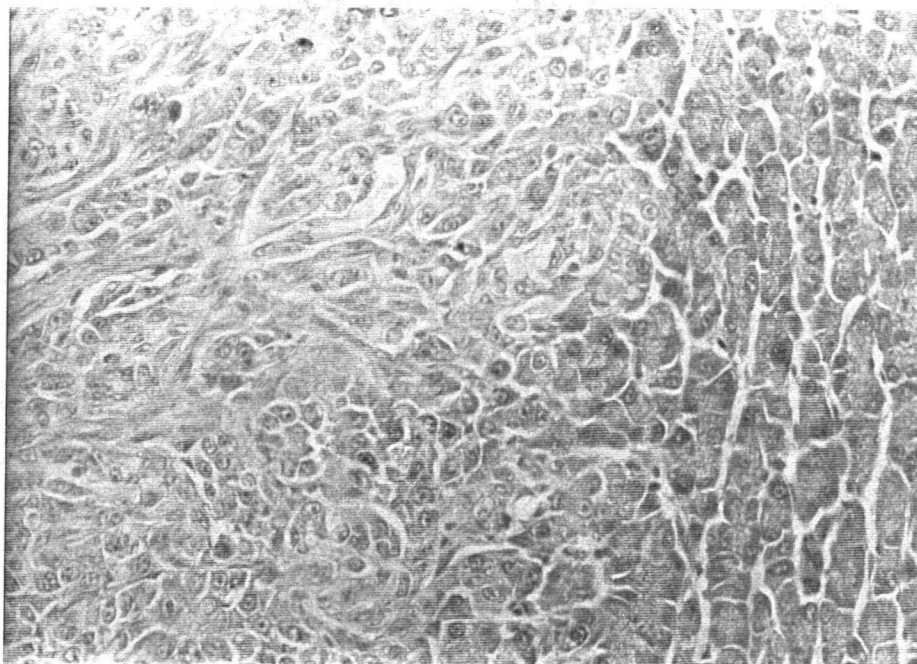


Fig. 2-5. Preneoplastic nodule in AFB₁-treated trout liver stained for GST- π (X 340). Hepatocytes surrounding nodule were stained positively for GST (right), and the cells in the foci were stained negatively for GST (left).

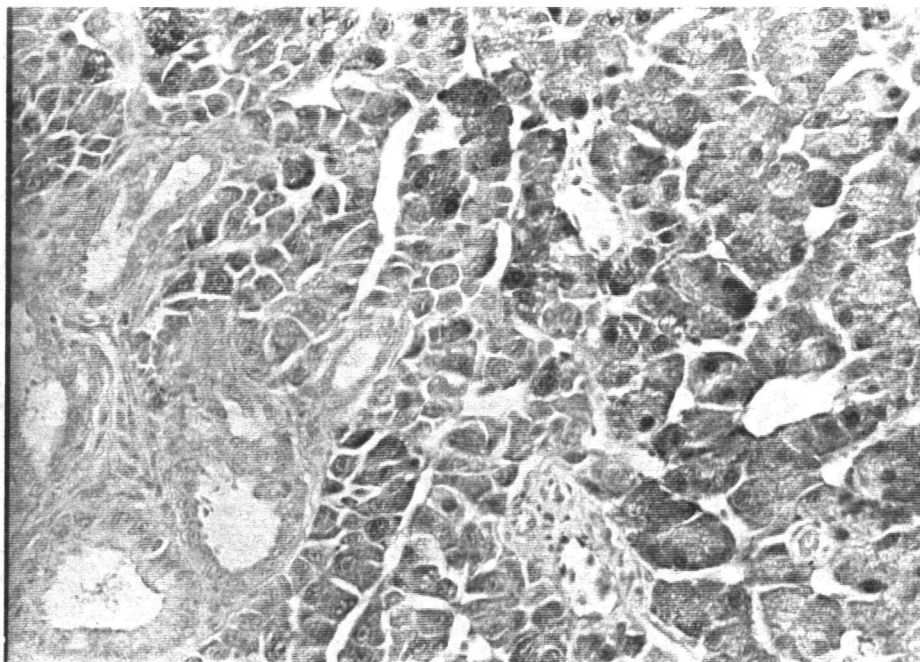


Fig. 2-6. Trout cholangioma stained for GST- π . Negative staining is demonstrated by the tumor cells (left) compared with the positive staining of the non-tumor cells (right) (X 340).

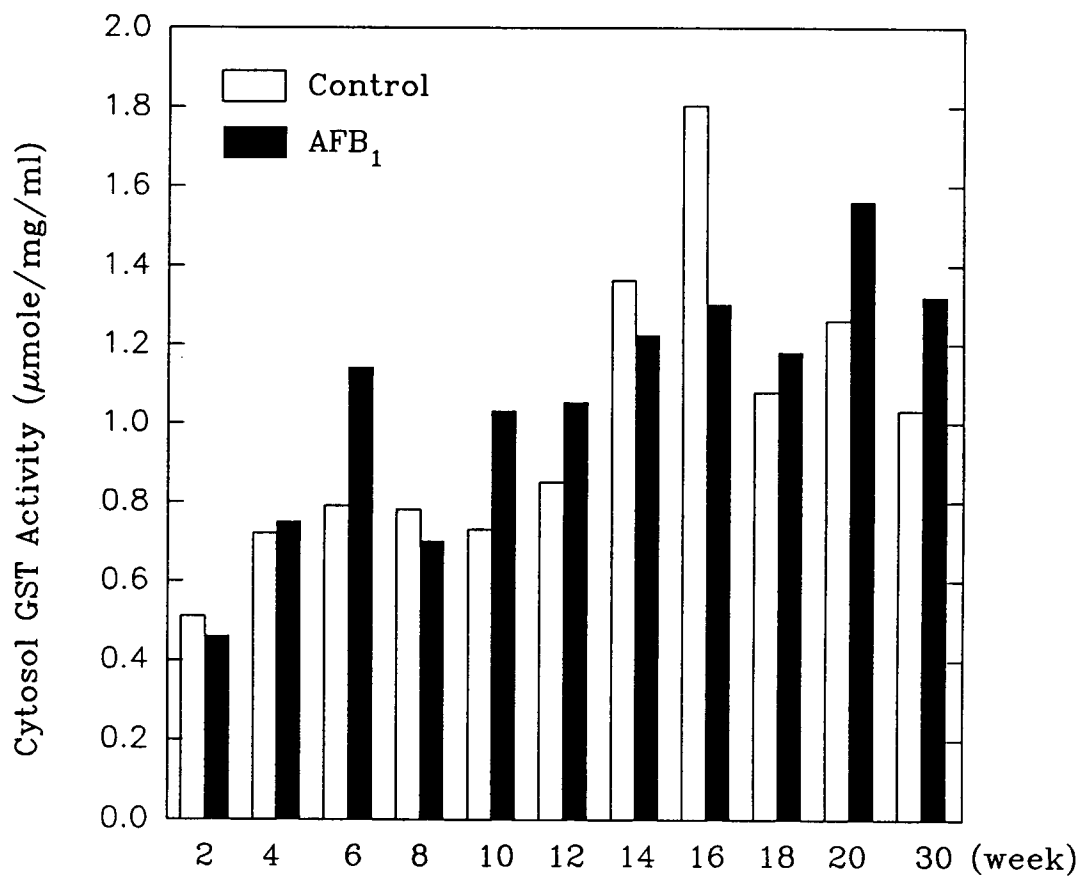


Fig. 2-7. Liver cytosolic GST activity in untreated trout (open bar), and AFB₁-treated trout (solid bar). X axis represents the time of sampling after treatment. Each value represents duplicate experiments for pooled liver samples from six fish.

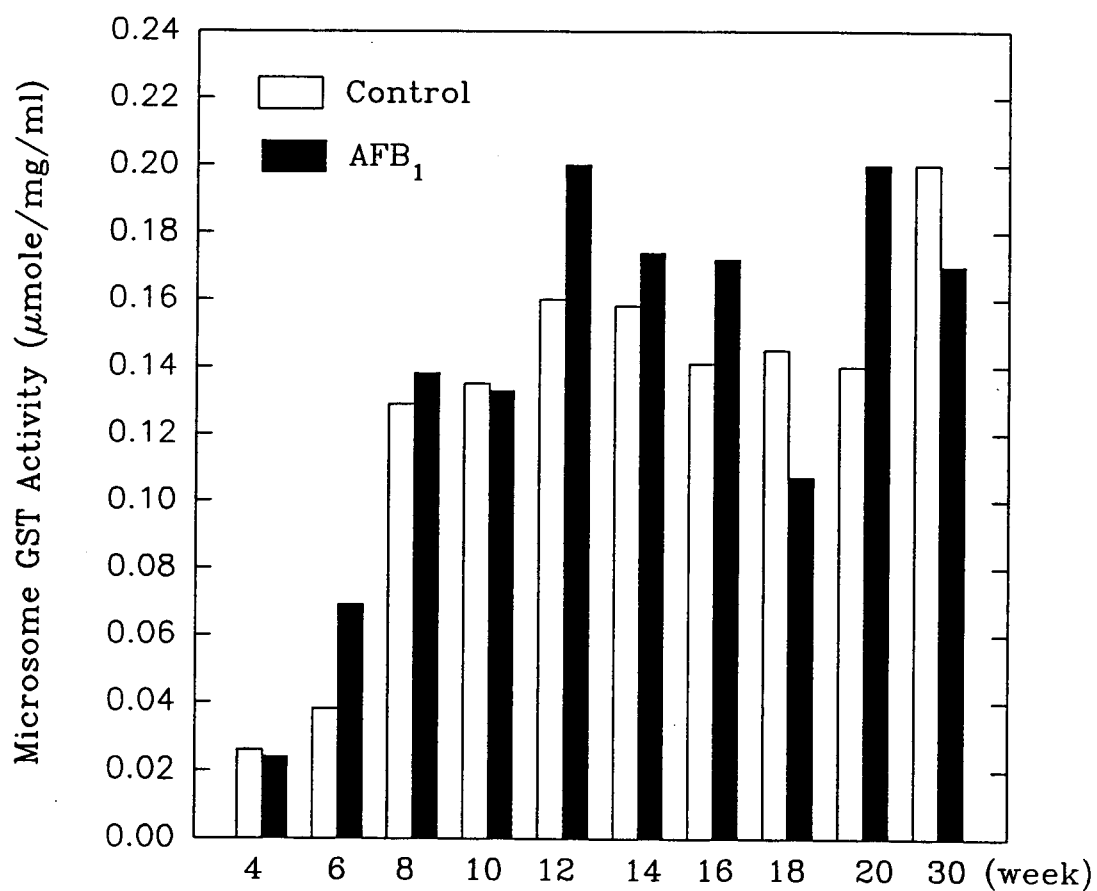


Fig. 2-8. Liver microsomal GST activity in untreated trout (open bar), and AFB₁-treated trout (solid bar). X axis represents the time of sampling after treatment. Each value represents duplicate experiments for pooled liver samples from six fish.

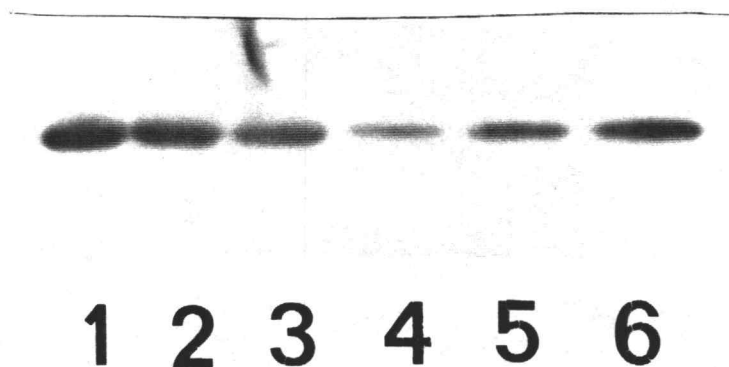


Fig. 2-9. Western blot of liver cytosolic protein isolated from control trout and AFB₁-treated trout using rabbit anti-trout GST- π antibody as a probe. Lane 1, 2, and 3 contained 20 μ g and were loaded with control sample, tumor sample, and non-tumor samples of treated fish respectively. Lane 4, 5, and 6 were purified trout GST, and contained 0.0625 μ g, 0.125 μ g, and 0.25 μ g respectively.

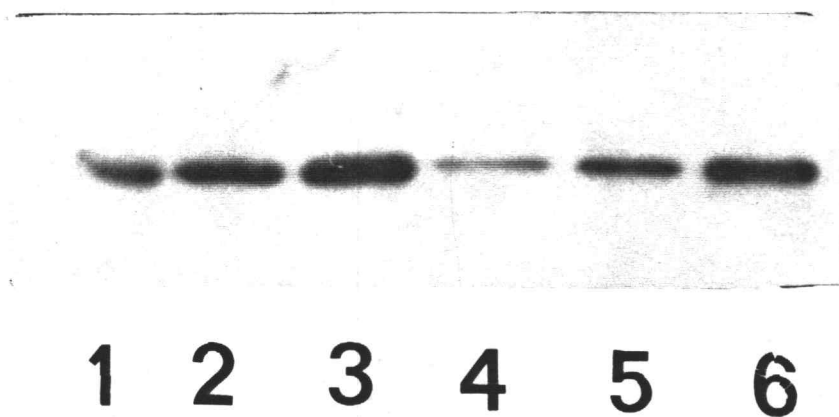


Fig. 2-10. Western blot of liver cytosolic protein isolated from control trout and AFB₁-treated trout using rabbit anti-human GST- π antibody as a probe. Lane 1, 2, and 3 contained 20 μ g and were loaded with control sample, tumor sample, and non-tumor samples of treated fish respectively. Lane 4, 5, and 6 were purified trout GST, and contained 0.0625 μ g, 0.125 μ g, and 0.25 μ g respectively.

Table 2-1. Hepatic GST activity of untreated and AFB₁-treated trout.

	GST activity (μ mole/mg/min)	
	Cytosol	Microsomes
Control	0.65	0.042
AFB ₁ -treated tumor	0.59	0.022
AFB ₁ -treated non-tumor	0.66	0.046

CONCLUSIONS

Nitrosamines are bioactivated in vivo to highly electrophilic metabolites capable of alkylating DNA as the initial step in carcinogenesis. In mammals, this reaction is primarily catalyzed by cytochrome P450 2E1. Although both mammals and rainbow trout are sensitive to the nitrosamine-induced carcinogenesis, PNP hydroxylase activity, a specific enzyme activity associated with cytochrome P450 2E1, was much lower in trout than that in the rat. Rainbow trout were refractory to the P450 2E1 inducers imidazole and ethanol. Following exposure of trout to 0.5 % ethanol, PNP hydroxylase activity of liver microsomes was slightly increased with increasing time of exposure in females but not in males. PNP hydroxylase activity in inbred trout were highest among three trout strains examined. There were four proteins, probably P450 isozymes, in the trout liver microsomes, that were recognized by rabbit anti-rat P450 2E1 IgG in Western blots. These proteins were P450 LMC1 and LMC2, a new P450 designated P450 X1 with an approximately M_r of 52,000 and a second new P450 isozyme, designated P450 X2, which comigrated with P450 LMC2 ($M_r = 54,000$). No correlation was observed between PNP hydroxylase activity and immunoquantitation of these isozymes. Although the level of P450 LMC2 in sexually mature males was higher than that of females, there was no gender difference in PNP hydroxylase activity. Our data suggests that P450 LMC2 is not responsible for the activation of nitrosamine in the trout. Whether other equivalent isozymes to P450 2E1 were involved in the nitrosamine-induced carcinogenesis in the trout remains to be established.

The glutathione S-transferase (GST) isolated from trout liver was classified as π family according to its N-terminal amino acid sequences and its dimeric composition. There was no difference in hepatic cytosolic and microsomal GST activity

between untreated and AFB₁-treated trout. The polyclonal antibodies raised against trout GST- π can be used as a biomarker for preneoplastic foci in AFB₁-treated rat liver. GST- π was expressed in rainbow trout normal liver but was lost in regenerating cells, preneoplastic lesions and neoplastic nodules in AFB₁-treated trout liver. Therefore, GST- π was a negative biomarker for preneoplastic nodules in AFB₁-induced carcinogenesis.

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